Iqbal Ahmad · Farah Ahmad
John Pichtel *Editors*

Microbes and Microbial Technology

Agricultural and Environmental Applications

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ISBN 978-1-4419-7930-8 e-ISBN 978-1-4419-7931-5
DOI 10.1007/978-1-4419-7931-5
Springer New York Dordrecht Heidelberg London

Library of Congress Control Number: 2011920994

Preface

Microbes are recognized for their ubiquitous presence, diverse metabolic activity, and unique survival strategies under extreme conditions. The diversity and abundance of microorganisms in various environments are poorly explored. However, rapid and continuous increase in global human population combined with rapid industrialization has resulted in environmental pollution with heavy metals, pesticides, and other toxic substances. Such impacts have adversely impacted crop production, the environment, and human health. Developing methods for sustaining crop production and environmental health are of prime importance in feeding global populations on a sustainable basis.

Molecular biology has revolutionized the study of microorganisms in the environment and improved our understanding of the composition, phylogeny, and physiology of microbial communities. The current molecular toolbox encompasses a range of DNA-based technologies, and new methods for the study of RNA and proteins extracted from environmental samples. Currently, there is a major emphasis on the application of "omics" approaches such as genomics, proteomics, functional genomics, etc. to determine the identities and functions of microbes inhabiting different environments. Recent molecular-based developments will be of significant value in discovering new microbes and microbial genes and to exploit them in solving the urgent challenges facing the environment, agriculture, and human health.

Emerging disciplines such as bioremediation, biofilms, microbial quorum sensing, and microbial nanoparticles require greater attention by researchers. Molecular techniques in tracking and monitoring microbial inoculants both in bulk soil and in rhizosphere are of critical value for bioinoculant efficacy monitoring. Therefore, exploring novel microbes and technologies are prerequisites for addressing the challenges of crop production and protection and environmental health management.

Considerable work has been carried out on the use of microbes in solving many agricultural and environmental pollution problems. A huge bank of data has already been generated on various practical aspects; however, the information is scattered and not available to all readers. There is a lack of concerted effort to publish edited books in this area and to address common agricultural and environmental problems where microbes could be efficiently applied to their management.

In recent years, the use of microbes and microbial technology has been considered for solving environmental pollution problems from heavy metals, pesticide contamination, etc. On the other hand, agricultural application to plant growth promotion and crop protection is not new; however, greater enthusiasm has appeared in recent years due to the urgency of maintaining sustainable crop productivity and the detection of deterioration of soil health. New dimensions such as probiotics, quorum sensing (i.e., cell-to-cell signaling), biofilms, and nanobiotechnology and their significance in environmental and agricultural issues embrace some of the recent trends of microbial technology.

The content of this book is divided into three main topic areas: microbial diversity exploration, new trends in research, and applications in the management of environmental pollution and protection of plant health. The book is divided into 18 chapters, with each focused on a specific topic to cover, diverse perspective topics. Topics include the exploration of microbial diversity and detection of microbial pathogens in food, concepts and applications of microbial biofilms, genetic exchange in bacterial populations in the natural environment, and classical and modern techniques for studying and tracking plant growth-promoting rhizobacteria. Recent developments in bioremediation of contaminated soil and water using microbial surfactants, bioaugmentation-assisted phytoremediation, degradation of agricultural pesticides by soil bacteria, biosorption of heavy metals and radionuclides by microbial biomass, recent trends in the role of baculoviruses and fungal-based agents in controlling plant pests and disease management, and production technology of mycorrhizal fungi are described. Current trends in the new frontiers of microbiology such as quorum sensing, biosensors, nanobiotechnology, and probiotics are also discussed in detail.

With contributions from a broad range of leading researchers, this book focuses on current trends in microbial diversity, detection, and microbial technology applications. Although aimed primarily at research scientists and graduate students in environmental and agricultural microbiology, the topics and techniques are equally applicable to all branches of microbial biotechnology.

With great pleasure, we extend our sincere thanks to all the learned contributors for their timely response, excellent contributions, and consistent support and cooperation. We express our deep sense of gratitude to all our respected teachers, scientific collaborators, colleagues, and friends for their guidance, support, and healthy criticism. The cooperation received from research students in book preparation is gratefully acknowledged. The names of selected students need special mention such as Mohammad Sajjad Ahmad Khan, Miss Maryam Zahin, and Fohad Mabood Husain.

It is not justified if we do not mention the inspiration/encouragement which we have received from many senior professors/scientists, especially Prof. R.J.C. McLean (USA), Prof. Hani Antoun (Canada), Prof. P.K. Wong (China), Dr. Vittorio Venturi (ICGEB, Italy), Dr. Elizabeth Grohmann (Germany), and Prof. M. Shamim Jairajpuri and Prof. M. Saleemuddin from AMU, Aligarh (India).

We must also offer special thanks to the colleagues/friends at AMU (Prof. Javed Musarrat, Prof. Akhtar Haseeb, Dr. M.R. Khan, Dr. Zaki Anwar Siddiqui, Dr. S. Hayat,

Dr. M. Owais, Dr. A. Malik, and Dr. M.S. Khan) for their encouragement, suggestions, and constructive criticism.

The technical assistance and support rendered from our excellent book publishing team, especially Dr. Melinda Paul and Meredith Clinton at Springer USA is most appreciated and acknowledged.

Many thanks to the members of our families for all the support they have provided.

Finally, we acknowledge Almighty God, who provided all the inspirations, insights, positive thoughts, and channels to complete this book project.

Aligarh, India — Iqbal Ahmad
Uttaranchal, India — Farah Ahmad
Muncie, IN — John Pichtel

About the Editors

Dr. Iqbal Ahmad, M.Phil., Ph.D. is a senior faculty in the Department of Agricultural Microbiology and has extensive postgraduate teaching experience since 1995 in agricultural microbiology, pathogenic microbes, disease and resistance to disease, antimicrobial drugs, biomolecules, food microbiology, molecular microbiology, and enzyme and fermentation technology. His research experience since 1989 encompasses various disciplines such as drug resistance and virulence in *E. coli* and plasmid biology, microbial ecology, impact of wastewater on soil health and crop productivity, drug and metal resistance in microbes of clinical and environmental origin, PGPR, bioactivities (i.e., antibacterial, antifungal, antioxidants, and antimutagenic) of medicinal plants and phytocompounds. His present research interest is in the fields of molecular basis of drug resistance and virulence factors linkage in pathogenic bacteria, bacterial quorum sensing, and modulation of quorum sensing-linked bacterial traits and biofilms by natural products. Dr. Ahmad has guided many Ph.D. and several M.Sc. theses and completed four research projects. His research works on Indian medicinal plants have received high citations. His edited books on various topics have been published by internationally reputed publishers. Dr. Ahmad is a member of various national and international scientific societies and serves as reviewer of several international and national journals.

Dr. Farah Ahmad, Ph.D. is graduated from Aligarh Muslim University and her expertise is mainly on soil and plant microbiology, with specific focus to plant growth promoting Rhizobacteria and completed her Ph.D. work with Dr. I. Ahmad, AMU, Aligarh. She has worked as Lecturer/Assistant Professor at the Department of Microbiology and Biotechnology, Integral University, Lucknow, India and King Khalid University, Abha, Saudi Arabia (KSA). Currently, she is actively engage in teaching and research in Microbiology, at the Sardar Bhagwan Singh Post Graduate Institute of Biomedical Sciences & Research Balawala, Dehradun (India). She has published/presented her research findings in scientific journals and presented in conferences. Her current research interest is in the fields of bioremediation and developing efficient bioinoculant for plant growth promotion and crop productivity.

Prof. John Pichtel, Ph.D., CHMM is a Professor of Natural Resources and Environmental Management at Ball State University in Muncie, IN, where he has been on the faculty since 1987. He received the Ph.D. degree in Environmental Science at Ohio State University, the M.S. degree in Soil Chemistry/Agronomy at Ohio State, and the B.S. degree in Natural Resources Management at Rutgers University. His primary research and professional activities have been in the management of hazardous materials, remediation of contaminated sites and environmental chemistry. Dr. Pichtel teaches courses in emergency response to hazmat incidents, management of solid and hazardous wastes, environmental site assessment, and site remediation.

Dr. Pichtel is a Certified Hazardous Materials Manager. He holds memberships in the Institute of Hazardous Materials Managers, Sigma Xi Scientific Society, and the Indiana Academy of Science. He was selected as a Fulbright Scholar in 1999 and again in 2005.

Dr. Pichtel has written three books addressing waste management and cleanup of contaminated sites and has been the author or coauthor of approximately 40 research articles. He has served as a consultant in hazardous waste management projects and has conducted environmental assessments and remediation research in the USA, the UK, Ireland, Finland, and Poland.

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Chapter 1
Microbial Applications in Agriculture and the Environment: A Broad Perspective

Iqbal Ahmad, Mohd Sajjad Ahmad Khan, Farrukh Aqil, and Mahipal Singh

Abstract Microbial diversity is an important component of the overall global biological diversity. Recent technological advances in exploring microbial diversity have revealed that a large proportion of microorganisms are still undiscovered, and their ecological roles are largely unknown. Careful selection of microbes and intelligent design of test assays are the key steps in developing new technologies for effective utilization of microorganisms for sustainable agriculture, environmental protection, and human and animal health. Several microbial applications are widely known in solving major agricultural (i.e., crop productivity, plant health protection, and soil health maintenance) and environmental issues (i.e., bioremediation of soil and water from organic and inorganic pollutants). Wastewater treatment and recycling of agricultural and industrial wastes are other important uses of microbial technology. It is expected that microbes in combination with developments in electronics, software, digital imaging, and nanotechnology will play a significant role in solving global problems of the twenty-first century, including climate change. These advances are expected to enhance sustainability of agriculture and the environment. This chapter provides an overview of recent trends in microbial exploitation in plant growth promotion and sustainable environment mainly through bioremediation, biodegradation, and biosorption processes. Recent uses and application of microbes such as biosensors, synthesis of nanomaterials, and probiotics are also discussed.

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_1,

1.1 Introduction

The microbial world is the largest unexplored reservoir of biodiversity on Earth. It is an important frontier in biology under intensive investigation. Interest in the exploration of microbial diversity has been prompted by the fact that microbes perform numerous functions essential for the biosphere that include nutrient cycling and environmental detoxification. The vast array of microbial activities and their importance to the biosphere and to human economies provide strong bases for understanding their diversity, conservation, and exploitation for society.

The environmental "super challenges" of the twenty-first century have become quite clear in the last several years. Climate change due to the vast increase in the production of greenhouse gases is real (Crowley 2000). There is a genuine need for renewable energy supplies (Cook et al. 1991; Jackson 1999). Constant threats of pandemics such as the Asian flu, Mad Cow disease, the outbreak of *Legionella* (Temmerman et al. 2006), as well as water shortages, shrinking agricultural productivity, and environmental contamination comprise some of the important issues. How can microbial resources address these challenges? A wide variety of microorganisms are present in soil, water, air, and in association with plants and animals. These diverse communities constitute "a metagenome of knowledge." This metagenome also extends to the microbial communities both inside and out of our body. Because of their metabolic actions, they are major players not only in our health and well-being but also in environmental sustainability (Verstaete et al. 2007).

Microbial culture collections currently contain more than one million different strains (http://www.wdcm.niq.ac.jp) and thus are testimony of the efforts made for the conservation of biodiversity and the desire to make these resources available to the public. To what extent these collections can and need to be expanded is debatable. It is generally accepted that microorganisms tend to act in association with others and not alone. It is, therefore, obvious that at present, considerable effort should be devoted to the collection and preservation of these novel microbial associations in natural samples as well as in enrichment cultures. In addition, preservation of the habitats in which these microbes thrive is needed. Until now, attention has mainly been focused on various unique sites such as hot springs and pristine locations (e.g., Arctic/Antarctic regions). The latter, for instance, has given rise over the past decade to an enormous knowledge of novel polar microbial taxa (van Trappen et al. 2005), which in turn has led to industrial applications such as cold-adapted enzymes (Siddiqui and Cavicchioli 2006), anti-freeze products (Gilbert et al. 2004), and many other strains capable of bioremediation in cold soils (Margesin et al. 2003). There is a need to explore more intensively new frontier habitats such as the deep oceans, the deep underground, endophytic microbes, and the deep intestine. Indeed, such environments harbor a wealth of putatively useful processes and products. Recent interesting discoveries include anaerobic ammonium oxidation (Anammox reaction) which converts ammonium and nitrite to dinitrogen gas in the sea (Kuypers et al. 2003), the Archaea-Bacteria consortia that oxidizes methane

anaerobically by means of sulfate reduction (Hinrichs et al. 1999; Boetius et al. 1997), and the pH 11–12-tolerant humus-degrading bacteria which occurs in the gut of soil-eating termites (Ji and Brune 2005). Not only are these "natural" habitats of value, but also a number of other sites, altered by industrial actions and often unwanted, are now to be earmarked as "resources" of microbial diversity. The best examples of these resources are sites exposed to acid mine drainage, which have recently delivered potential anticancer drugs (Yamada et al. 2004) and aquifers polluted with chloroorganics, which have yielded very interesting halo-respiring microorganisms (de Wildeman and Verstraete 2003; Smidt and de Vos 2004). Thus, selection of specific unexplored microbial habitats, natural or modified, may be of great significance in terms of benefits for the environment, agriculture, and society.

1.2 Approaches to Studying Soil Microbial Populations

Soil is considered a storehouse of microbial activity. Living microorganisms are estimated to comprise less than 5% of the total space occupied. Therefore, major microbial activity is confined to "hot spots" i.e., aggregates with accumulated organic matter, rhizosphere (Pinton et al. 2001). Soil microbial communities are often difficult to characterize, mainly because of their immense phenotypic and genotypic diversity, heterogeneity, and crypticity. With respect to latter, bacterial populations in top layers of the soil profile can produce over 10^9 cells/g soil (Torsvik and Ovreas 2002). Most of these cells are unculturable. The fraction of the cells making up soil microbial biomass that have been cultured and studied in detail are negligible and are often less than 5% of the total population (Torsvik et al. 1990; Borneman and Triplett 1997; Ovreas and Torsvik 1998). The soil may be studied for microbiological, biochemical, and functional diversity using various approaches (Paul 2007).

Methods of studying microbial diversity can be broadly divided into two categories: (1) cultivation-based methods and (2) cultivation-independent methods. Both approaches have their unique limitations and advantages (Garbeva et al. 2004).

1.2.1 Cultivation-Based Methods

Traditional methods to study microbial diversity were based on cultivation and isolation of microbes (van Elsas et al. 1998). A wide variety of culture media have been formulated to maximize the variety and populations of microorganisms. A Biolog™-based method for directly analyzing the potential activity of soil microbial communities displaying community level physiological profiling (CLPP) has been used to study microbial diversity (Garland 1996).

1.2.2 Cultivation-Independent Methods

Recent developments in molecular technology have helped to better understand soil microbial diversity. These molecular techniques include polymerase chain reaction (PCR) and real-time polymerase chain reaction (RT-PCR), which are used to target the specific DNA or RNA in soil. The 16S or 18S ribosomal RNA (rRNA) or their genes (rDNA) represent useful markers for prokaryotes and eukaryotes, respectively. PCR products generated with primers based on conserved regions of the 16S or 18S rDNA using total DNA or RNA of the specific soil microbial community yield a mixture of DNA fragments representing all PCR accessible species present in the soil sample. The mixed PCR products can be used for (1) preparing clone libraries (Borneman and Triplett 1997; McCaig et al. 1999) and (2) a range of microbial community fingerprinting. Such clone libraries are useful for identification and characterization of the dominant bacterial or fungal types in soil and thereby provide a picture of microbial diversity (Garbeva et al. 2004). Moreover, a range of other techniques have been developed to fingerprint soil microbial communities. For instance, denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) (Heur et al. 1997; Muyzer and Smalla 1998), amplified rDNA restriction analysis (ARDRA) (Massol-Deya et al. 1995), terminal restriction fragment length polymorphism (T-RFLP) (Liu et al. 1997), single-stranded conformational polymorphism (SSCP) (Schmalenberger and Tebbe 2002), and ribosomal intergenic spacer analysis (RISA) (Ranjard and Richaume 2001) have been applied with great success.

1.3 Functional Diversity of Microbes

Functional diversity is the most important parameter for characterization and exploitation of microbial cultures. Similarly, functional genomics are considered powerful tools for discovering novel functions associated with an organism's genome. Depending upon the target use of the organism, they have been given different names which indicate their major functions in nature or under defined conditions. However, to obtain a novel class of compounds and functions, an intelligent design of test system and careful selection of microbes is a prerequisite for a successful screening strategy. The wild strain obtained from various reservoirs may further be subjected to strain improvement programs (mutation, genetic exchange, protoplast fusion, and gene regulation) to increase the productivity and/or fitness of the culture in a specific location (Crueger and Crueger 2003). An overview of the possible use of microorganisms is elaborated in Fig. 1.1.

1.4 Application in Agriculture and the Environment

Various microbes of soil and other origins have been widely studied and exploited in crop production, crop protection, soil health improvement, and compost preparation. Microbial products have also been exploited in controlling

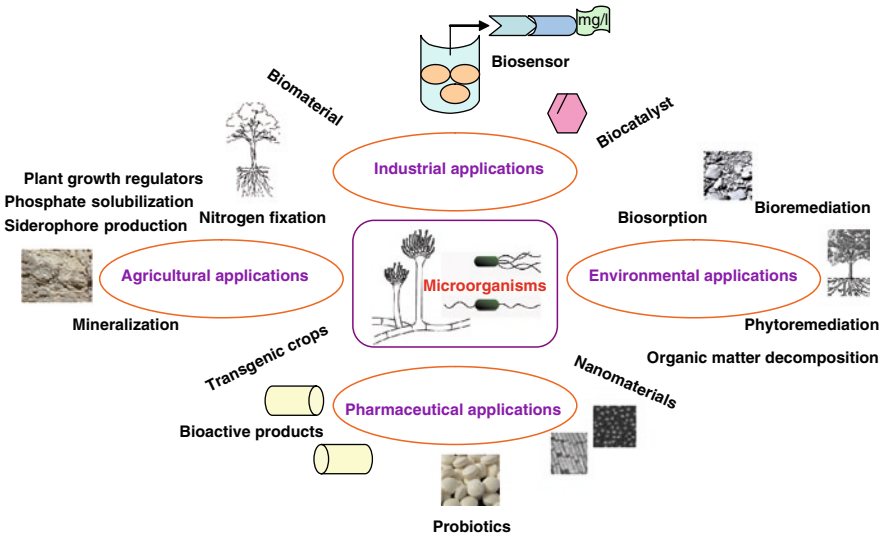

Fig. 1.1 Scope and applications of microbial products

plant and animal diseases. Recent developments in microbial and plant molecular biology have made it possible to develop transgenic plants with improved gene delivery systems. Many successful examples of transgenic crops are now available. On the other hand, use of microorganisms in industrial, pharmaceutical and food industries are enormous and beyond the scope of the present article. Similarly, environmental exploitation for bioremediation of soil, water, and other polluted habitats with organic and inorganic pollutants are well known and extensively documented in the literature. However, due to the lack of information on microbial diversity of various unique extreme habitats and poor understanding of nonculturable microorganisms, novel approaches are needed to explore and utilize the untapped microbial diversity in agriculture, the environment, and human health. In addition to classical uses and application of microbes, new dimensions have been explored where microbes are expected to provide solutions to specific problems and applications. Some are briefly discussed here.

1.4.1 Microbes in Plant Growth Promotion and Health Protection

Plant-pathogenic microorganisms are a major and chronic threat to food production and ecosystem stability worldwide. As agricultural production has intensified over the past few decades, producers have become more and more dependent on agrochemicals as a relatively reliable method of crop protection, which ultimately

imparts economic stability to their operations. However, increasing use of chemical inputs causes several negative effects. Today, the concepts of integrated plant nutrient and integrated plant disease and pest management must be perfected according to resources available and agroclimatic and economic conditions. In this approach, use of biological agents (biofertilizers, biopesticides, biocontrol agents, and PGPRs) is an integral part of the management. Biocontrol is thus being considered as alternative or a supplemental means of reducing the use of chemicals in agriculture (de Weger et al. 1995; Gerhardson 2002; Postma et al. 2003, Welbaum et al. 2004). There is a large body of literature describing potential uses of plant-associated bacteria as agents stimulating plants, and managing soil and plant health (Glick 1995; Hallman et al. 1997; Rovira 1965; Sturz et al. 2000; Welbaum et al. 2004). The term plant growth-promoting bacteria (PGPB) was coined in 1978 by Kloepper and Scroth. Most PGPRs are members of fluorescent Pseudomonas (Glick 1995). PGPB as well as plant growth-promoting fungi, both symbiotic and free-living in the rhizosphere, are associated with many, if not all, plant species and are present in many environments. The most widely studied group of PGPBs are plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978), which colonize root surfaces and the closely adhering soil interface, the rhizosphere [Kloepper and Schroth 1978; Kloepper et al. 1999, as reviewed by Kloepper et al. (1999) or, more recently, by Gray and Smith (2005), and Ahmad et al. (2008a, b)]. The nature of these PGPR varies from free-living to endophytic, diazotrophs to non-diazotrophs and other symbiotic fungi.

The widely recognized mechanisms of biocontrol mediated by PGPBs involve competition for an ecological niche or a substrate, production of inhibitory allelochemicals, and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens (Glick 1995; Haas et al. 2000; Bloemberg and Lugtenberg 2001; Lugtenberg et al. 2001; Haas et al. 2002) and/or abiotic stresses (Nowak and Shulaev 2003). Research into the mechanisms of plant growth promotion by PGPB has provided a greater understanding of the multiple facets of disease suppression by these biocontrol agents. Still, most of the focus has been on free-living rhizobacterial strains, especially *Pseudomonas* and *Bacillus*. Much remains to be learnt from nonsymbiotic endophytic bacteria that have unique associations and apparently a more pronounced growth-enhancing effect on host plants (Conn et al. 1997; Chanway et al. 2000; Bais et al. 2004). Revelations about the mechanisms of PGPB action open new doors to design strategies for improving the efficacy of biocontrol agents (Walsh et al. 2001; Morrissey et al. 2002, 2004). Similarly, modulation of the rhizosphere bacterial consortia can be accomplished by soil aeration, hydrogenation, and delivery of molasses, sugars and by appropriate crop rotations (Welbaum et al. 2004). Identifying different mechanisms of action facilitate the combination of strains, bacteria with bacteria or bacteria with fungi, to hit pathogens with a broader spectrum of microbial weapons (Duffy et al. 1996; Leeman et al. 1996; Schisler et al. 1997; Pierson et al. 1998; Raupach and Kloepper 1998; de Boer et al. 1999; Kilic-Ekici and Yuen 2004; Lutz et al. 2004; Olivain et al. 2004). Along this same line, biotechnology can be applied to further improve strains that possess valued qualities (e.g., ease of formulation, stability, or otherwise those exceptionally

suited to plant colonization) by creating transgenic strains that combine multiple mechanisms of action (Timms-Wilson et al. 2000; Chin-A-Woeng et al. 2001; Huang et al. 2004). For example, transforming the1-aminocyclopropane-1-carboxylic acid deaminase gene, which directly stimulates plant growth by cleaving the immediate precursor of plant ethylene (Glick et al. 1998.) into *P. fluorescens* CHAO, not only increases plant growth but can also increase biocontrol properties of PGPB (Wang et al. 2000). Continued work with endophytic bacteria also holds potential for developing biocontrol agents that may be self-perpetuating by colonizing hosts and being transferred to progeny, as is the case with associative nitrogen-fixing PGPB on sugarcane (Boddey et al. 2003) or the nonsymbiotic endophyte bacterium *Burkholderia phytofirmans* (Nowak and Shulaev 2003; Sessitsch et al. 2005). Performance of a developed microbial agent should also be assessed in integrated plant nutrition management for effective utilization.

A number of other bacteria are now considered to be PGPR, including species of *Azotobacter*, *Azospirillum*, *Acetobacter*, *Burkholderia*, and Bacilli. Virtually any nondeleterious free-living bacteria which could promote plant growth directly or indirectly may be designated as PGPR (Glick 1995; Glick et al. 1999). Recently, various other PGPR have been identified including *Achromobacter, Arthobacter, Azocarus, Clostridium, Enterobacter, Flavobacterium, Frankia, Hydrogenophaga, Kluyvera, Microcoleous, Phyllobacterium, Serratia, Staphylococcus, Streptomyces*, and *Vibrio* (Bashan and de-Bashan 2005; Ahmad et al. 2008).

PGPR may promote plant growth either directly or indirectly. Direct mechanisms include (1) the ability to produce the plant growth regulators (indoleacetic acid, gibberellins, cytokinins, and ethylene); (2) asymbiotic N_2 fixation; and (3) solubilization of mineral nutrients such as phosphates. Indirect mechanisms involve (1) antagonism against phytopathogens; (2) production of siderophores; (3) production of extracellular cell wall degrading enzymes of phytopathogens such as β-1, 3-glucanase, chitinase; (4) antibiotic production; and (5) cyanide production as described by Ahmad (2006) and Ahmad et al. (2008).

1.4.1.1 Plant Growth-Promoting Fungi

The role of various plant-associated microbes is widely known, for instance, legume–rhizobium interaction, role of mycorrhiza in plant growth promotion, etc. Free-living fungi have also been involved in the promotion of plant growth by one or another mechanism. One such example is by phosphate solubilization. These organisms are popularly known as phosphate solubilizers. The role of such organisms was widely studied in the 1980–1990s in India and other parts of the world (Gaur 1990). However, due to the discovery of other environmental benefits associated with these organisms, new interest has been shown by many workers (Khan et al. 2009). More recently, the role of free-living fungi screened from Indian soil for their multiple potential PGP activities by Imran (2010) at our laboratory indicated that many phosphate-solubilizing fungi possess many additional beneficial traits, including production of plant growth hormones, many extracellular

enzymes, resistance to many toxic metals, biosorption ability for Ni, Cd, and Cr, and also a contribution to plant growth enhancement when used as inoculants for wheat and chickpea under field conditions. Thus, efforts should be directed toward the exploration of additional organisms for better exploitation in crop productivity and environmental pollution management.

1.4.2 Microbes in Environmental Problem Management

Environmental pollutants in soil and water are a major concern worldwide. Many toxic, mutagenic, and carcinogenic elements pose serious threats to the environment and public health. Contaminated water and wastewater can be treated by means of chemical, physical, and biological means to remove and/or detoxify it. Similarly, various methods such as thermal desorption and landfilling can be used to treat contaminated soil. But these soil treatments do not effectively restore natural flora and fauna. Bioremediation, i.e., the use of microorganisms to remove toxic pollutants from the environment, is the most promising technology that is eco-friendly, safe, and effective even if the pollutants are present at low concentrations (as in the case with heavy metal removal from water) (Labana et al. 2005; Singh 2006; Zafar et al. 2007; Lal et al. 2010).

Many site-specific microorganisms are capable of carrying out bioremediation reactions, and many have already been used at sites previously contaminated with polycyclic aromatic hydrocarbons (PAHs), nitroaromatic compounds, chlorinated organics, etc. (Samanta et al. 2002; Zocca et al. 2004; Carvalho et al. 2005). In many cases, the contaminants are not completely mineralized and their derivatives may accumulate and create their own unique health hazards (Singh 2006). To find a solution to this problem, various strategies are considered including the use of various combination of microorganisms with capabilities for mineralizing certain forms of the pollutants and its derivatives. An excellent review article by Lal et al. (2010) indicated the potential and prospects of microflora in rapid degradation of pesticides as benzene hexachloride and related compounds. Another avenue, which is essential in this direction, is the exploration and/or engineering of new catabolic pathways and study of regulatory control of primary and secondary metabolites, to generate effective bioremediation reactions. This is a difficult task, as we do not possess complete information of in situ bacterial adaptation to environmental stresses and regulation of various metabolic genes. However, the development of bacterial genomics, proteomics, and metabolomics plus the development of sophisticated new techniques in medical sciences make it possible to explore global protein expression and low molecular weight metabolite expression (metalbolomics) in environmental bioremediation (Singh 2006).

Bioremediation involves the utilization of organisms or derivatives from organisms to degrade pollutants. The chief advantage of bioremediation is its reduced cost compared with conventional techniques such as incineration for which the remediation of all contaminated sites in the USA alone is estimated at $1.7 trillion

(Kuiper et al. 2004), or $7,000 per citizen. In addition, bioremediation often provides a permanent solution (providing complete transformation of the pollutant to its molecular constituents such as carbon dioxide and water) rather than a method that simply transfers wastes from one phase to another (Kuiper et al. 2004). Biological catalysts have enormous catabolic potential for remediating wastes; however, the interactions between bacteria and pollutants are often complex and suitable remediation does not always take place. Moreover, many man-made compounds lack good biological catalysts (for most of the ten million organic compounds described, biodegradation has not been investigated, and in many instances good biocatalysts fail to transform pollutants in the environment). Hence, the field remains a fertile area for application of new biotechnological methods to facilitate bioremediation, such as metabolic engineering, proteomics, reverse genetics, transcriptomics, metabolomics, and genome-scale metabolic modeling. In addition, follow-up studies are important for determining why pollutants persist. Metabolic engineering involves redirecting the cell's metabolism to achieve a particular goal using recombinant engineering (Bailey 1991). One of the first and finest examples of this approach in bioremediation was the metabolic engineering of *Pseudomonas* sp. B13; five different catabolic pathways from three different bacteria were combined to allow for degradation of methylphenols and methylbenzoates in a single organism (Rojo et al. 1987). Ju and Parales (2009) enabled bacteria, for the first time, to utilize chloronitrobenzenes for growth without the addition of cosubstrates and create the first strain that grows on 3-chloronitrobenzene. Chloronitrobenzenes are manufactured for pesticides, fungicides, dyes, and polymers. The bacteria accomplish this feat by cleverly introducing an enzyme that removes nitro groups, nitrobenzene 1,2-dioxygenase from *Comamonas* sp. strain JS765, into *Ralstonia* sp. strain JS705, a strain that has an ortho pathway for the degradation of chlorocatechols. The authors carefully show that 3-chloronitrobenzene is converted by the cloned nitrobenzene 1,2-dioxygenase into 4-chlorocatechol (with release of nitrite) which is subsequently degraded by the host *Ralstonia* sp. strain JS705. They also utilize an active-site mutant of the large subunit of the dioxygenase (F293Q) to reduce the doubling time on 3-chloronitrobenzene by 25%.

Related to the degradation of nitroaromatic compounds by microbes is the article by Fernandez et al. (2009), which shows that the model bacterium *Pseudomonas putida* KT2440 can grow in the presence of saturated concentrations of the widely used nitroaromatic explosive, 2,4,6-trinitrotoluene. Using DNA microarrays, transposon mutants, and isogenic mutants, the authors found that the organism reacts to the compound via activation of a series of detoxification functions including nitroreductase, isoquinolone oxidoreductase, dehydrogenase, and chaperones to prevent or repair cell damage. The authors also show that multidrug efflux pump genes (*mexEF*/*oprN*) are induced to reduce intracellular trinitrotoluene concentrations. This work is groundbreaking in that few groups have applied transcriptomics to bioremediation, and this technique promises to help unravel unforeseen regulatory bottlenecks related to successful remediation. Matilla et al. (2007) also used whole-transcriptome profiling to determine mutualistic interactions in the rhizosphere for strains relevant for bioremediation; for example, 90 rhizosphere

upregulated genes were identified for *P. putida* growing on corn roots. Further in the line of subsurface contamination, Scheibe et al. (2009) present a genome-based metabolic model of the metabolism of *Geobacter sulfurreducens* and couple this to a hydrological transport model to predict in situ uranium bioremediation. As *Geobacter* activity to reduce U (VI) is critically dependent on the availability of acetate as an electron donor and Fe (III) as an electron acceptor (plus ammonium as key nutrient), predictive modeling clearly helps to discover the limiting factors and concentrations under natural environmental conditions. The model accurately predicted the behavior of *Geobacter* in a field trial of uranium bioremediation, demonstrating the power of coupling genome-scale metabolic models with hydrological models for field-scale behavior. Further insights into the rates of intrinsic bioremediation, that of microbial degradation of hydrocarbon subsurface contaminants under anaerobic conditions at two fuel-contaminated sites, are provided by Gieg et al. (2009). Using deuterated compounds and skilful analytical work, they show that the long lag phases (weeks to months) seen in many laboratory experiments may not adequately predict the fate of these fuel contaminants as they measure lags of hours to days for a wide range of compounds; hence, these pollutants may be degraded far more rapidly than predicted. Evidence for anaerobic bioremediation of a wide range of compounds including toluene, *m*-xylene, ethylbenzene, 1,3,5,-trimethylbenzene, and hexane includes identification of degradation intermediates involving fumarate as well as other intermediates.

1.4.2.1 PAH Degradation

PAHs that possess more than three aromatic rings have been referred to as high molecular weight (HMW) PAHs in the environmental microbiology literature. The physical and chemical properties of HMW PAHs are such that they generally appear to be persistent in the environment and may pose risks to human and ecological health in parent molecule form or after biological and/or chemical transformations (Lundstedt et al. 2007). HMW PAHs are sparingly soluble in water, are electrochemically stable, and may be acutely toxic, genotoxic, immunotoxic (Burchiel and Luster 2001), or act as agents of hormone disruption (van de Wiele et al. 2005), depending upon circumstances and mode of exposure. Due to their elevated octanol–water partition coefficients (*K*ow), HMW PAHs may partition into organic phases, soil and sediment organic matter, and membranes of living organisms and are candidates for bioconcentration, bioaccumulation, and sometimes biomagnification through trophic transfers in terrestrial and marine food webs (Neff 2002; Meador 2003). The environmental levels of HMW PAHs vary widely; they appear to be ubiquitous in the environment; their occurrence has been studied in the atmosphere (Lang et al. 2008), soil (Nam et al. 2009), freshwater and marine sediments (Zakaria et al. 2002), ice cores (Kawamura and Suzuki 1994), in the deep oceans (Ohkouchi et al. 1999), and in numerous other media ranging from vegetation to food (Wagrowski and Hites 1997; Fismes et al. 2002).

Interest in understanding prokaryotic biotransformation of HMW PAHs has continued to grow and the scientific literature shows that studies in this field

originate from research groups from many different locations throughout the world. In the last 10 years, research in regard to HMW PAH biodegradation by bacteria has been further advanced through the documentation of new isolates that represent diverse bacterial types that have been isolated from different environments and that possess different metabolic capabilities. This has occurred in addition to the continuation of in-depth comprehensive characterizations of previously isolated organisms such as *Mycobacterium vanbaalenii* PYR-1.

New metabolites derived from prokaryotic biodegradation of four- and five-ring PAHs have been characterized. Knowledge of the enzymes involved in these transformations has been advanced and HMW PAH biodegradation pathways have been further developed, expanded upon, and refined. At the same time, investigation of prokaryotic consortia has furthered our understanding of the capabilities of microorganisms functioning as communities during HMW PAH (Kanaly and Harayama 2010).

1.4.2.2 Microbes in Metal Removal from Water

A recent development in environmental microbial technology involves the use of microbe-based sorbents for removal and recovery of strategic and precious heavy metals from industrial wastewater. Various microorganisms including bacteria, fungi, algae, and yeast have been subjected to intense scrutiny for their potential to remove heavy metals from aqueous solutions by active and passive mechanisms. Progress made in the last 2 decades indicates that the biosorption process can replace conventional processes of heavy metal pollution control or at least may be more effective in bioremediation in combination with other techniques.

1.4.2.3 PGPR in Biomanagement of Metal Toxicity

The improper disposal, misuse, and accidental release of toxic and organic and inorganic compounds into the environment have resulted in widespread pollution of soil, groundwater, and marine environments. As the adverse environmental and health effects of these materials become better known, increasing attention is being directed toward the development and implementation of innovative technologies for cleaning up this contamination (Hopper 1989). Contamination of agricultural soil with heavy metals has been increasing largely due to disposal of improperly treated wastewater and sewage, and agricultural runoff in many developing and developed countries. Such contamination has exerted adverse effects, both on soil health and crop productivity. PGPB may be useful in reducing the toxicity of metals to plants. This phenomenon could occur in two ways. The use of ACC deaminase-containing PGPB could decrease stress ethylene in plants growing in metal-enriched soil. In addition, plants are able to take up and utilize complexes of bacterial siderophores and iron. Plant siderophores bind to iron with a much lower affinity than bacterial siderophores do, so in metal-contaminated soils, a plant is unable to accumulate sufficient iron unless bacterial siderophores are also present (Glick 2003; Sylvia et al. 2005).

A plant growth-promoting bacterium, *Kluyvera ascorbata* SUD165 that contained high levels of heavy metals, was isolated from soil collected near Sudbury, Ontario, Canada. The bacterium was resistant to the toxic effects of Ni^{2+}, Pb^{2+}, Zn^{2+}, and CrO_4^- and produced a siderophore(s), and displayed 1-aminocyclopropane 1-carboxylic acid deaminase activity. Canola seeds inoculated with this bacterium and grown under gnotobiotic conditions in the presence of high concentrations of nickel chloride were partially protected against nickel toxicity. In addition, protection by the bacterium against nickel toxicity was evident in pot experiments with canola and tomato seeds. The presence of *K. ascorbata* SUD165 had no measurable influence on the amount of nickel accumulated per milligram (dry weight) of either roots or shoots of canola plants. Therefore, the bacterial plant growth-promoting effect in the presence of nickel was probably not attributable to the reduction of nickel uptake by seedlings. Rather, it may reflect the ability of the bacterium to lower the level of stress ethylene induced by the nickel (Burd et al. 1998).

The siderophore-overproducing mutant *K. ascorbata* 165/26 exerted a more pronounced effect on plant growth than did the wild-type bacterium *K. ascorbata* SUD 165. These bacteria had the ability to protect plants against the inhibitory effects of high concentrations of nickel, lead, and zinc; it was hypothesized that this effect was related to bacteria providing plants with sufficient iron (Burd et al. 2000).

Rajkumar et al. (2005) isolated RNP_4 obtained from a long-term tannery waste-contaminated soil, which was characterized and presumptively identified as *Pseudomonas* sp. The strain RP_4 tolerated concentrations up to 450 mg Cr^{6+}/L on Luria-Britani (LB) agar medium and reduced substantial amounts of Cr^{6+} to Cr^{3+} in the medium. Furthermore, the strain was able to promote the growth of black gram, Indian mustard, and pearl millet in the presence of Cr^{6+}. Thus, the innate capability of two novel isolates for parallel bioremediation and plant growth promotion had significance in the management of environmental and agricultural problems.

1.5 Microbial Biosensors and Their Applications

A biosensor is an analytical device that combines a biological sensing element with a transducer to produce a signal proportional to analyte concentration (Mulchandani and Rogers 1998; Mikkelson and Corton 2004). This signal can result from a change in proton concentration, release or uptake of gases, light emission, absorption, and so forth brought about by cellular metabolism. The transducer converts this biological signal into a measurable response such as current, potential or absorption of light that can be amplified, processed, and stored for later analysis (Mulchandani and Rogers 1998). The biological sources for biosensors include enzymes, antibodies, receptors, organelles, and microorganisms. Cells of higher organisms such as those from animals and plants or tissue have also been

used. Among these, biological sources from microorganisms are more widely used. Microorganisms have been integrated with a variety of transducers such as amperometric, potentiometric, calorimetric, conductimetric, colorimetric, luminescent, and fluorescent to construct biosensor devices (Ramsay 1998; D'Souza 2001). Considerable scientific literature addressing microbial biosensor development has been published (Belkin 2003; Lei et al. 2006). Enzymes are the most widely used biological sensing element in the fabrication of biosensors (Mulchandani and Rogers 1998; Mikkelson and Corton 2004); purified enzymes are very specific, but the process is costly and tedious. Microorganisms provide an ideal alternative, but of less specificity compared to enzymes. Recent progress in molecular biology and RDT have opened new possibilities of tailoring microorganisms to improve the activity of an existing enzyme or express foreign enzymes/proteins in a host cell (Belkin 2003; Rensing and Maier 2003). The basis of a microbial biosensor is the close contact between microorganisms and the transducer. Thus, fabrication of a microbial biosensor requires immobilization on a transducer in proximity. Immobilization technology, therefore, plays an important role. There are several physical and chemical methods to immobilize the microorganism on transducer or support matrices (Mulchandani and Rogers 1998; Mikkelson and Corton 2004). Details of these methods and their advantages and limitations can be seen in the literature (Lei et al. 2006). Table 1.1 summarizes various types of biosensors along with some examples. Microbial biosensors can be classified, based on the transducers, into electrochemical, optical, and others.

A variety of biosensors have been developed for environmental, food, military, and biomedical applications. This is primarily due to their low cost, long lifetime, and wide range of suitable pH and temperature ranges. However, when compared to enzyme biosensors, the development of highly satisfied microbial sensors is still hampered because they suffer from long response time, low sensitivity, and less selectivity. Recent developments in microbial genomics and DNA technology have given hope to the development of microbial biosensors for extreme conditions and with more specific applications in the future.

1.6 Microbes and Nanoparticles

Nanomaterials are at the leading edge of the rapidly developing field of nanotechnology. Use of microbes in synthesizing nanoparticles comes under the broad topic of microbial or bionanotechnology. This is a multi-interdisciplinary area where experts from physics, chemistry, biology, and engineering must act in a coordinated manner. There is an enormous interest in the synthesis of nanomaterials due to their unique optical, chemical, and electronic properties (Kumar et al. 2003). Recent developments in the organization of nanoscale structures into predefined superstructures ensure that nanotechnology will play a significant role in the new millennium in areas such as catalysis, biomedicine, mechanics, magnetic, and energy sciences.

Table 1.1 Various kinds of biosensors currently in use

Biosensors	Target	Microorganism	Limit of detection	References
Amperometric microbial biosensors	BOD	*A. adeninivorans* LS3	1.24 mg/L	Chan et al. (2000)
		P. putida	0.5 mg/L	Chee et al. (1999)
		K. oxytoca AS1	<44 mg/L	Ohki et al. (1994)
		B. subtilis	2–22 mg/L	Riedel et al. (1988)
		Pseudomonas sp.	1–40 mg/L	Li and Chu (1991)
		P. fluorescens	15–260 mg/L	Yoshida et al. (2001)
	Ethanol	*A. aceti* (IFO 3284)	<0.2 mM	Ikeda et al. (1997)
		G. suboxydans	0–25 mg/L	Kitagawa et al. (1987)
		C. tropicalis	0.5–7.5 mM	Akyilmaz and Dinckaya (2005)
		A. niger	1–32 ppm	Subrahmanyam et al. (2001)
		G. oxydans	1.1–2.2 g/L	Tkac et al. (2000)
	Cyanide	*T. ferrooxidans*	0.5 μM	Okochi et al. (2004)
	Phenolic compounds	*P. putida*	0.5–6 μM, 0.3–2.5 μM and 0.02–0.2 μM	Timur et al. (2003)
Potentiometric microbial biosensors	Organophosphates	*Flavobacterium* sp.	0.025–0.4 mM	Gaberlein et al. (2000)
		Recombinant *E. coli*	2 μM	Mulchandani et al. (1998)
Bioluminescence and fluorescence microbial biosensors	Ni^{2+} and Co^{2+}	*Ralstonia eutropha* AE2515	0.1 μM Ni^{2+}, 9 μM Co^{2+}	Tibazarwa et al. (2001)
	Bioavailable mercury	*E. coli* HMS174 harboring *mer-lux* plasmid pRB27	10 pM	Rasmussen et al. (1997)
		E. coli HMS174 harboring *mer-lux* plasmid pRB28	nM level	Selifonova et al. (1993)
	Pollution-induced stress	*P. fluorescens* pUCD607	~2 ppm	Porteous et al. (2000)

Partially adapted from Lei et al. (2006)

The synthesis of nanomaterials over a range of chemical compositions and high monodispersity is still a challenge in materials science. Several manufacturing techniques that usually employ atomistic, molecular, and particulate processing in a vacuum or a liquid medium are in use (Daniel and Astruc 2004). Most techniques are capital-intensive as well as inefficient in materials and energy use. Hence, there is an ever-growing need to develop clean, nontoxic, and environmentally benign synthesis procedures. Consequently, researchers in nanoparticle synthesis have turned to biological systems for inspiration. It is well known that many organisms can provide inorganic materials either intra- or extracellularly (Simkiss and Wilbur 1989; Mann 1996). For example, unicellular organisms such as magnetotactic bacteria produce magnetite nanoparticles (Lovley et al. 1987; Spring and Schleifer 1995; Dickson 1999), and diatoms synthesize siliceous materials (Mann 1993; Oliver et al. 1995; Kroger et al. 1999). Multicellular organisms produce hard inorganic–organic composite materials such as bones, shells, and spicules using inorganic materials as a foundation to build complex structures (Lowenstam 1981).

Biominerals are composite materials and consist of an inorganic component and a special organic matrix (proteins, lipids, or polysaccharides) that controls the morphology of the inorganic compound. The surface layer bacteria produce gypsum and calcium carbonate layers (Pum and Sleytr 1999; Sleytr et al. 1999). Even though many biotechnological applications such as remediation of toxic metals employ bacteria (Stephen and Macnaughton 1999) and fungi (Mehra and Winge 1991), such microorganisms have recently been found to be potential eco-friendly nanofactories. Processes devised by nature for the synthesis of inorganic materials on nano- and microlength scales have contributed to the development of a relatively new and largely unexplored area of research based on the use of microbes in the biosynthesis of nanomaterials (Sastry et al. 2004). Below we provide a brief overview of the current research worldwide on the use of bacteria and actinomycetes (both prokaryotes), as well as algae, yeast, and fungi (eukaryotes) in the biosynthesis of metal nanoparticles and their applications (Mandal et al. 2006).

1.6.1 Fungi in Nanoparticle Synthesis

The use of fungi in the synthesis of nanoparticles is a relatively recent addition to the list of potentially relevant microorganisms. The use of fungi is potentially exciting since they secrete large amounts of enzymes and are simpler to deal with in the laboratory. However, the genetic manipulation of eukaryotic organisms, as a means of overexpressing specific enzymes identified in nanomaterial synthesis, is more difficult than that of prokaryotes. An extensive screening process resulted to two genera, which, when challenged with aqueous metal ions such as $AuCl_4^-$ and Ag^+, yielded large quantities of metal nanoparticles either extracellularly (Mukherjee et al. 2002; Ahmad et al. 2003) or intracellularly (Mukherjee et al. 2001a, b). The appearance of a distinctive purple color in the biomass of *Verticillium* after exposure to the 10^{-4} M $HAuCl_4$ solution indicated the formation

of gold nanoparticles intracellularly (Mukherjee et al. 2001a). At higher magnification, the 5- to 200-nm-sized nanoparticles with an average size of 20 ± 8 nm were clearly seen populating both the cell wall and the cytoplasmic membrane of the fungal cell. Furthermore, the powder diffraction pattern recorded from the biofilm indicated the crystalline nature of gold nanoparticles. The exposure of *Verticillium* sp. to silver ions resulted in a similar intracellular growth of silver nanoparticles (Mukherjee et al. 2001b).

The use of microorganisms such as bacteria, yeasts, algae, fungi, and actinomycetes in the biosynthesis of metal nanoparticles has been now defined and described. This interdisciplinary field, "bionanotechnology," requires collaboration between physicists, chemists, biologists, and engineers. A number of issues need to be addressed from the nanotechnology and microbiology points of view before such biosynthetic procedures can compete with traditional protocols. The elucidation of biochemical pathways, leading to metal ion reduction among the different classes of microbes, is necessary to develop a rational microbial nanoparticle synthesis procedure. The surface chemistry of biogenic nanoparticles should be properly recognized as well. Genetic engineering techniques can potentially be used to improve particle properties and control their composition. The shift from bacteria to fungi as a means of developing natural "nanofactories" has the added advantage that downstream processing and handling of the biomass would be much simpler. At present, microbial methods in the synthesis of nanomaterials of varying composition are extremely limited and confined to metals, some metal sulfides, and very few oxides. An extension of the procedures to enable reliable synthesis of nanocrystals of other oxides (TiO_2, ZrO_2, etc.), nitrides, and carbides could make microbial synthesis a commercially feasible proposition.

1.7 Other New Applications

Various examples illustrate the progress, based on novel discoveries in microbial ecology, which open up new applications and research in microbial technology. Some of these applications are briefly described.

1.7.1 Microbes and Climate Change

The anthropogenic production of carbon dioxide is currently of major concern to scientists, national representatives, and the public. Yet it represents only 10% of that of the CO_2 produced normally by soil. Sustainable agriculture permits the buildup of humus in the soil (i.e., carbon sequestration) on the order of 0.3–1.0 tons C per ha per year. This way, about 10% of all carbon emitted by automobiles can be compensated by applying quality agriculture. In addition, microbial dynamics as a function of temperature and pCO_2 are not yet fully understood. There is a

possibility that changes in climate and land use can be compensated for by the "homeostasis" of the microbial communities.

The role of soil microbes in both the formation and removal of CO_2 and NO_2 is well known. Similarly, in the case of methane, it has been established that the rumen microbiology is responsible for some 30% of all emissions and novel research about short-circuiting rumen methanogens by providing alternate electron acceptors such as herbal components are underway. Rice paddies are a second important emitter of methane (approx. 30% of the global contribution), and an intriguing potential consists of trapping the reducing equivalents in the mud layer by electrodes (van De Woestyne et al. 1994). Third, in terms of methane emissions, are waste landfills (about 15% contribution), and here modern biotechnology of anaerobic digestion offers the possibility to capture these emissions and use them to produce utilizable energy (Boeckx et al. 1997). Most significant of all, however, are the services rendered to abatement of methane by the methanotrophic microbes present in soils. These bacteria scavenge on the order of 800–1,000 kg CH_4 per ha per year (Mohanty et al. 2006). Understanding their role and the extent of their capacities is clearly warranted. Moreover, one should be capable to prevent that they are inhibited by the use of fertilizers and pesticides (Keppler et al. 2006) and even consider seeding and enhancing them on sites of concern such as methane production by the forest phylosphere (Feijtel et al. 1985). There are a number of novel perspectives and applications in the domain of biohydrogen/bioethanol/biodiesel/biogas and bioelectricity generation. One can even speculate about the potential of harvesting the sun's by means of the combined route of legumes coupled to hydrogen-producing *Rhizobium* symbionts (Rabaey and Verstraete 2005). Proposals to harvest sediments, wastes, and biorefinery downstreams by means of biogas technology coupled to microbial fuel cell technology will also continue to generate interest (Aeltermann et al. 2006).

1.7.2 Probiotics and Health

The Russian Nobel Prize winner Elie Metchnikoff first suggested that certain bacteria could modify the composition of the gut flora (Metchnikoff 1907). He suggested that the longevity of Bulgarians and Russians of the Steppes was due to their consumption of "sour milk" containing beneficial microbes, which in fact are probably lactic acid bacteria (LAB) such as *Lactobacillus bulgaricus*. Henry Tissier of the Pasteur Institute isolated bacteria (now called *Bifidobacterium bifidum*) from the feces of healthy breast-fed infants and recommended giving it to babies suffering from diarrhea (Tissier 1900). In 1935, Minoru Shirota in Japan developed the first commercial probiotic drink called Yakult, which contains *Lactobacillus casei* Shirota that can survive the passage through the stomach and colonize the intestine. The probiotic market is now estimated to be worth about $6,000,000,000 per year and is growing at about 10% annually (UBIC-Consulting 2008). Since 1981, there have been over 2,000 patent applications on probiotics filed (with "probiotic"

mentioned somewhere in the patent) and some 524 granted (in the USA and Europe). The two most commonly used probiotics in commercial products are lactobacilli, members of the LAB, and bifidobacteria. However, some yeast and other bacteria have also been claimed to have probiotic potential (Ouwehand et al. 2002). For an overview of commercially used strains and their claimed probiotic effects and genomics of these strains, see Siezen and Wilson (2010).

Probiotics are most commonly known as yogurts or yogurt-type drinks. The consumption of probiotics by humans is intended to improve or maintain a healthy intestine. The claimed modes of action of probiotics, include strengthening of the intestinal barrier function, modulation of immune responses, supply of vitamins, and antagonism of pathogens (or other commensals). For recent reviews, readers are directed to articles of Ventura et al. (2007), Kalliomaki et al. (2008), Lebeer et al. (2008), Kleerebezem and Vaughan (2009), and Siezen and Wilson (2010).

From an industrial perspective, crucial attributes of probiotic strains are good technological properties for production and storage, and low health risk to consumers. Probiotics need not be restricted to food applications or oral delivery. Some can be applied to the skin as lotions or cream (Krutmann 2009) and have been used to treat vaginal infections (Reid 2008). Probiotics are also added to animal and fish feed to enhance growth, replacing banned additive antibiotics or growth hormones (Gatesoupe 2008; Higuchi et al. 2008; Wynn 2009). They appear to work by inhibiting/reducing the pathogenic bacterial load that some animals or fish carry. There is ample evidence for all of the above probiotic modes. Important microorganisms exploited for probiotics include: *Bacillus coagulans* GBI-30, *Bifidobacterium animalis* ssp. *Lactis* HN019 (DR10), *Bifidobacterium infantis* 35624, *Bifidobacterium longum* BB536, *Escherichia coli* M-17, *Escherichia coli* Nissle 1917, *Lactobacillus acidophilus* DDS-1, *Lactobacillus acidophilus* LA-5, *Lactobacillus brevis* KB290, *Lactobacillus casei* DN114-001, *Lactobacillus paracasei* St11, *Lactobacillus johnsonii* NCC533 LC1, *Lactobacillus plantarum* 299v, *Lactobacillus rhamnosus* GR-1, *Lactobacillus reuteri* RC-14, *Bifidobacterium bifidum* BB-12, and *Lactobacillus helveticus* R0052 as described by Siezen and Wilson (2010).

Publicly available complete genome sequences of putative probiotic bacteria which include members of *Bifidobacterium* and *Lactobacillus* are available in the GOLD database (http://www.genomesonline.org).

At present, many of the commercial probiotic strains originate from the intestines of healthy infants and adults. Current research focuses on the determination of the characteristics that these bacteria employ to survive and compete successfully in the intestine. With this knowledge, more effective probiotic strains can be identified. To speed up this search, numerous gut metagenomic sequencing efforts are ongoing worldwide to identify potential new probiotic candidates (Gill et al. 2006; Kurokawa et al. 2007). See also the Human Gut Metagenome Initiative (http://www.international.inra.fr/press/mapping_the_human_intestinal_metagenome) and the Human Gut Microbiome Initiative (Gordon et al. 2006) (http://genomeold.wustl.edu/hgm/ HGM_frontpage.cgi).

Perhaps the future will bring us health-promoting drinks containing mixtures of many probiotic strains, much like the cocktails used today, for vaccination against

infectious diseases. And what will be the next hype? Memory enhancing drinks would perhaps be a commercial success on quiz nights in the pub as suggested by Siezen and Wilson (2010).

1.8 Conclusion

With the rapid increase of world population, there is a consequent increase in the rate of resource utilization and environmental perturbation. On the other hand, there is a greater need to sustain and increase agricultural productivity and human health. Unexplored microbial diversity and available culturable microbes are the main bioresource to be exploited to solve the major challenges of twenty-first century. The genetic potential of various extreme habitats are considered to be useful for industrial technology. Future research and extension will go a long way toward applications of microbes for the improvement of environmental quality, agricultural productivity, human health and, for novel uses such as global climate change, nanomaterials, biosensors, biofuels, and probiotics.

Our future efforts should be directed toward (1) exploration of various unexplored habitats of microbial resources; (2) exploitation of plant health, plant genome promotion, and bioremediation research; (3) metagenomics, functional microbial genomics, and novel applications in sustainability of the environment; and (4) the role of microbes in global climate change, crops, new drugs development, and transgenic development.

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Chapter 2
Molecular Techniques to Assess Microbial Community Structure, Function, and Dynamics in the Environment

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Abstract Culture-based methods are important in investigating the microbial ecology of natural and anthropogenically impacted environments, but they are extremely biased in their evaluation of microbial genetic diversity by selecting a particular population of microorganisms. With recent advances in genomics and sequencing technologies, microbial community analyses using culture-independent molecular techniques have initiated a new era of microbial ecology. Molecular analyses of environmental communities have revealed that the cultivable fraction represents <1% of the total number of prokaryotic species present in any given sample. A variety of molecular methods based on direct isolation and analysis of nucleic acids, proteins, and lipids from environmental samples have been discovered and revealed structural and functional information about microbial communities. Molecular approaches such as genetic fingerprinting, metagenomics, metaproteomics, metatranscriptomics, and proteogenomics are vital for discovering and characterizing the vast microbial diversity and understanding their interactions with biotic and abiotic environmental factors. This chapter summarizes recent progress in the area of molecular microbial ecology with an emphasis on novel techniques and approaches that offer new insights into the phylogenetic and functional diversity of microbial assemblages. The advantages and pitfalls of commonly used molecular methods to investigate microbial communities are discussed. The potential applications of each molecular technique and how they can be combined for a greater comprehensive assessment of microbial diversity has been illustrated with example studies.

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_2,

2.1 Introduction

The biosphere is dominated by microorganisms and contains about $4–6 \times 10^{30}$ prokaryotic cells (Whitman et al. 1998). This number represents at least two to three orders of magnitude more than all of the plant and animal cells combined. Thus, microorganisms are highly diverse group of organisms and constitute about 60% of the Earth's biomass (Singh et al. 2009). In aquatic environments, such as the oceans, the number of microbial cells has been estimated to be approximately 1.2×10^{29}, while in terrestrial environments, soil sustains as many as $4–5 \times 10^{30}$ microbial cells (Singh et al. 2009). Owing to such enormous numbers, microorganisms are essential components of the Earth's biota and represent a large unexplored reservoir of genetic diversity. Understanding this unexplored genetic diversity is a high-priority issue in microbial ecology from perspectives such as global climate change and the greenhouse effect.

Microorganisms are key players in important ecological processes such as soil structure formation, decomposition of organic matter and xenobiotics, and recycling of essential elements (e.g., carbon, nitrogen, phosphorous, and sulfur) and nutrients. Thus, microbes play a critical role in modulating global biogeochemical cycles and influence all lives on Earth (Garbeva et al. 2004). In fact, all organisms in the biosphere either directly or indirectly depend on microbial activities. In soil ecosystems, microorganisms are pivotal in suppressing soil-borne plant diseases, promoting plant growth, and in promoting changes in vegetation (Garbeva et al. 2004). An understanding of microbial dynamics and their interactions with biotic and abiotic factors is indispensable in bioremediation techniques, energy generation processes, and in biotechnological industries such as pharmaceuticals, food, chemical, and mining.

The three fundamental questions that exist while discovering and characterizing any natural or artificial ecosystem are the following: (1) what type of microorganisms are present? (2) what do these microorganisms do? and (3) how do the activities of these microorganisms relate to ecosystem functions (e.g., energy flow, biogeochemical cycling, ecological resilience)? Microbial ecology aims to answer these central questions and deals with the study of microorganisms and their interactions with each other and with their environment. A plethora of biochemical and molecular methods have been applied to reveal the microbial community composition over time and space in response to environmental changes. These new approaches allow linkage between ecological processes in the environment with specific microbial populations and help us to answer important questions in microbial ecology such as what factors and resources govern the enormous genetic and metabolic diversity in an environment. This chapter presents an overview of the potentials and limitations of current molecular approaches used in microbial ecology. Although these techniques have been discussed with special emphasis on soil and plant microbial ecosystems, these are equally applicable to many other environments as well, such as oceans and sediments.

2.2 Culture Methods in Microbial Ecology: Applications and Limitations

Standard culture techniques to characterize microbial ecology involve isolation and characterization of microorganisms using commercial growth media such as Luria–Bertani medium, Nutrient Agar, and Tryptic Soy Agar (Kirk et al. 2004). The major limitation of culture-based techniques is that >99% of the microorganisms in any environment observed through a microscope are not cultivable by standard culturing techniques (Hugenholtz 2002). Several improved cultivation procedures and culture media have been devised that mimic natural environments in terms of nutrients (composition and concentration), oxygen gradient, pH, etc. to maximize the cultivable fraction of microbial communities. For example, a technique has been devised for the cultivation of uncultured microorganisms from different environments including seawater and soil that involved encapsulation of cells in gel microdroplets for large-scale microbial cultivation under low nutrient flux conditions (Zengler et al. 2005). Nonetheless, not all "uncultured" organisms are cultivable, and many of them remain "unculturable." These organisms, although viable in their natural environments, do not grow under laboratory conditions and remain in a "viable but nonculturable" (VBNC) stage (Oliver 2005). Such VBNC organisms could represent completely novel groups and may be abundant or very active but remain untapped by standard culture methods.

Molecular microbial surveys based on 16S rRNA genes reveal that candidate bacterial divisions such as BRC1, OP10, OP11, SC3, TM7, WS2, and WS3 have no cultured representatives and are known only by their molecular sequences (Schloss and Handelsman 2004). These division-level clades, such as OP11, are highly diverse and widely distributed in different environments and are considered as "candidate divisions" to reflect our limited knowledge due to the lack of any cultured representative. Studies suggest the existence of at least 50 bacterial phyla with half represented entirely by molecular sequences (Schloss and Handelsman 2004). Additionally, microorganisms retrieved using common culture methods are rarely numerically abundant or functionally significant in the environment from which they were cultured. These cultured microorganisms are considered as the "weeds" of the microbial world and constitute <1% of all microbial species (Hugenholtz 2002). For example, most of the isolates cultured from soil samples belong to one of four phyla (the "big four"), Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, primarily due to their ease of cultivation under laboratory conditions. Although Acidobacteria constitutes on average 20% of soil bacterial communities, these organisms are difficult to culture and are represented by few genera (Schloss and Handelsman 2004). These findings suggest that molecular techniques that circumvent the need for isolation and cultivation are highly desirable for in-depth characterization of environmental microbial communities.

2.3 Molecular Methods of Microbial Community Analyses

The vast majority of microbial communities in nature have not been cultured in the laboratory. Therefore, the primary source of information for these uncultured but viable organisms is their biomolecules such as nucleic acids, lipids, and proteins. Culture-independent nucleic acid approaches include analyses of whole genomes or selected genes such as 16S and 18S rRNA (ribosomal RNA) for prokaryotes and eukaryotes, respectively. Based on the comparative analyses of these rRNA signatures, cellular life has been classified into three primary domains: one eukaryotic (Eukarya) and two prokaryotic (Bacteria and Archaea) (Hugenholtz 2002). Over the last few decades, the field of microbial ecology has seen tremendous progress, and a wide variety of molecular techniques have been developed for describing and characterizing the phylogenetic and functional diversity of microorganisms (Fig. 2.1). Broadly, these techniques have been classified into two major categories depending on their capability of revealing the microbial diversity structure and function: (1) partial community analysis approaches and (2) whole community analysis approaches.

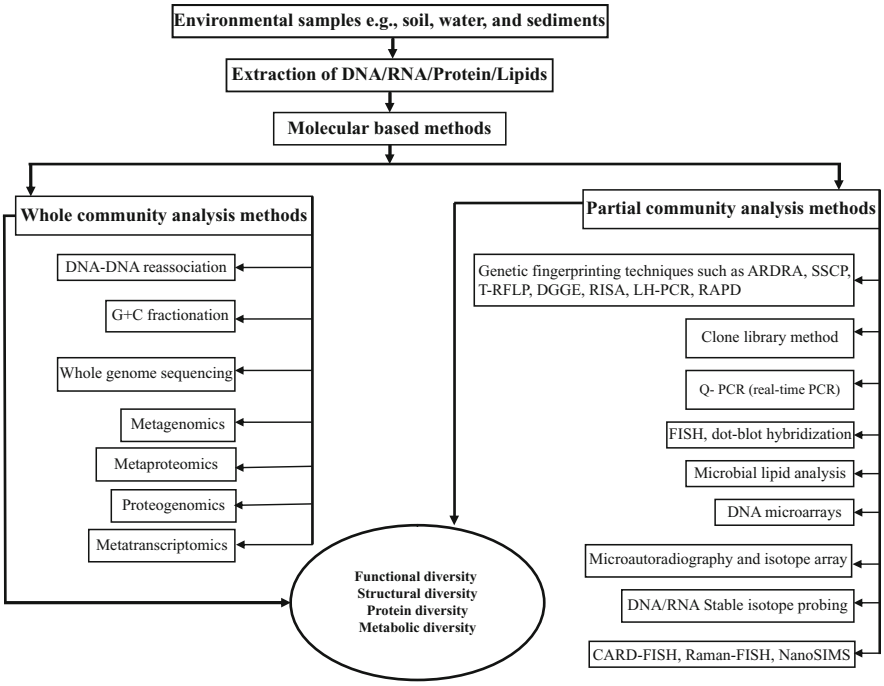

Fig. 2.1 Culture-independent molecular toolbox to characterize the structural and functional diversity of microorganisms in the environment

2.3.1 Partial Community Analysis Approaches

These strategies generally include polymerase chain reaction (PCR)-based methods where total DNA/RNA extracted from an environmental sample is used as a template for the characterization of microorganisms. In principle, the PCR product thus generated reflects a mixture of microbial gene signatures from all organisms present in a sample, including the VBNC fraction. PCR amplification of conserved genes such as 16S rRNA from an environmental sample has been used extensively in microbial ecology primarily because these genes (1) are ubiquitous, i.e., present in all prokaryotes, (2) are structurally and functionally conserved, and (3) contain variable and highly conserved regions (Hugenholtz 2002). In addition, the suitable gene size (~1,500 bp) and growing number of 16S rRNA sequences available for comparison in sequence databases make it a "gold standard" choice in microbial ecology. By estimating the phylogenetic relatedness to known microorganisms based on the homology of 16S rRNA sequences, the closest affiliation of a new isolate or molecular sequence is assigned. Other conserved genes such as RNA polymerase beta subunit (*rpoB*), gyrase beta subunit (*gyrB*), recombinase A (*recA*), and heat shock protein (*hsp60*) have also been used in microbial investigations and to differentiate some bacterial species (Ghebremedhin et al. 2008). The PCR products amplified from environmental DNA are analyzed primarily by (1) clone library method, (2) genetic fingerprinting, (3) DNA microarrays, or by a combination of these techniques.

2.3.1.1 Clone Library Method

The most widely used method to analyze PCR products amplified from an environmental sample is to clone and then sequence the individual gene fragments (DeSantis et al. 2007). The obtained sequences are compared to known sequences in a database such as GenBank, Ribosomal Database Project (RDP), and Greengenes. Typically, cloned sequences are assigned to phylum, class, order, family, subfamily, or species at sequence similarity cut-off values of 80, 85, 90, 92, 94, or 97%, respectively (DeSantis et al. 2007). While clone libraries of 16S rRNA genes permit an initial survey of diversity and identify novel taxa, studies have shown that environmental samples like soil may require over 40,000 clones to document 50% of the richness (Dunbar et al. 2002). However, typical clone libraries of 16S rRNA genes contain fewer than 1,000 sequences and therefore reveal only a small portion of the microbial diversity present in a sample. A cloning-and-sequencing method was used to decipher the microbial community composition in mining-impacted deep subsurface soils of the former Homestake gold mine of South Dakota, USA (Rastogi et al. 2009). Phylogenetic analysis of 230 clone sequences could reveal only a partial view of phylogenetic breadth present in soil samples. Rarefaction analyses of clone libraries generated nonasymptotic plots, which indicated that diversity was not exhaustively sampled due to insufficient clone sequencing, a common problem when assessing environmental microbial

diversity using cloning approaches. Despite its limitations (e.g., labor-intensive, time-consuming, and cost factor), clone libraries are still considered the "gold standard" for preliminary microbial diversity surveys (DeSantis et al. 2007). With the advent of newer and inexpensive sequencing methods, great progress is expected in this method of microbial diversity analysis.

2.3.1.2 Genetic Fingerprinting Techniques

Genetic fingerprinting generates a profile of microbial communities based on direct analysis of PCR products amplified from environmental DNA (Muyzer 1999). These techniques include DGGE/TTGE, SSCP, RAPD, ARDRA, T-RFLP, LH-PCR, RISA, and RAPD and produce a community fingerprint based on either sequence polymorphism or length polymorphism. In general, genetic fingerprinting techniques are rapid and allow simultaneous analyses of multiple samples. Fingerprinting approaches have been devised to demonstrate an effect on microbial communities or differences between microbial communities and do not provide direct taxonomic identities. The "fingerprints" from different samples are compared using computer-assisted cluster analysis by software packages such as GelCompar, and community relationships are inferred. Community fingerprints are scored as present or absent, and the similarities among samples are determined using Jaccards' coefficient.

Denaturing- or Temperature-Gradient Gel Electrophoresis

In denaturing-gradient gel electrophoresis (DGGE), the PCR products are obtained from environmental DNA using primers for a specific molecular marker (e.g., 16S rRNA gene) and electrophoresed on a polyacrylamide gel containing a linear gradient of DNA denaturant such as a mixture of urea and formamide (Muyzer et al. 1993). Temperature-gradient gel electrophoresis (TTGE) is based on the same principle of DGGE except that a temperature gradient rather than chemical denaturant is applied. Sequence variation among different PCR amplicons determines the melting behavior, and therefore amplicons with different sequences stop migrating at different positions in the gel. Both DGGE and TTGE involve the use of a 5′-GC clamped (30–50 nucleotides) forward primer during the PCR step. This is essential to prevent the two DNA strands from complete dissociation into single strands during electrophoresis. For determining the phylogenetic identities from DGGE/TGGE fingerprints, the bands can be excised from the gel, reamplified, and sequenced or blotted onto nylon membranes and hybridized to molecular probes specific for different taxonomic groups. DGGE profiles generated using universal bacterial primers from soil microbial communities are generally very complex. In order to overcome this problem, group-specific PCR-DGGE with primers targeting only specific physiological/phylogenetic groups has been used (Mühling et al. 2008). The other problems associated with DGGE/TGGE are as follows: (1) limited sequence information (<500 bp) obtained for phylogenetic analysis from DNA

bands, (2) different DNA fragments may have similar melting points, (3) number of different DNA fragments, which can be separated by polyacrylamide gel electrophoresis (PAGE), and (4) sequence heterogeneity among multiple rRNA operons of one bacterium, leading to multiple bands in DGGE, which might overestimate the diversity. DGGE analysis has been used to screen the unique clones in clone libraries based on distinct patterns and determining the number of operational taxonomic units (OTUs). In a microbial community investigation, DGGE was applied to soils collected from different agricultural fields in Norway and the USA that were under different agronomic treatments (crop rotation and tillage) (Nakatsua et al. 2000). Of these soil samples, one was also highly contaminated by polyaromatic hydrocarbons (PAH, 700 mg kg^{-1}). DGGE profiles were generated using primers based on V3 and V6/V9 regions for the bacterial population and V3 region of 16S rRNA for archaeal communities. Results showed that bacterial diversity was far greater than archaeal diversity except for the PAH-contaminated soil sample.

Single-Strand Conformation Polymorphism

In single-strand conformation polymorphism (SSCP), the environmental PCR products are denatured followed by electrophoretic separation of single-stranded DNA fragments on a nondenaturing polyacrylamide gel (Schwieger and Tebbe 1998). Separation is based on subtle differences in sequences (often a single base pair), which results in a different folded secondary structure leading to a measurable difference in mobility in the gel. Unlike DGGE, SSCP technology does not require any GC clamped primers, gradient gels, or specialized electrophoretic apparatus; therefore, it is a more simple and straightforward technique than DGGE. Similar to DGGE, the DNA bands can be excised from the gel, reamplified, and sequenced. However, SSCP is well suited only for small fragments (between 150 and 400 bp) (Muyzer 1999). A major limitation of the SSCP method is the high rate of reannealing of DNA strands after an initial denaturation during electrophoresis, which can be overcome using a phosphorylated primer during PCR, followed by specific digestion of the phosphorylated strand with lambda exonuclease. SSCP has successfully been employed to differentiate the pure cultures of *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Sinorhizobium meliloti* isolated from soil samples (Schwieger and Tebbe 1998). These authors have also applied SSCP for the analysis of rhizosphere bacterial communities associated with two different plant species, *Medicago sativa* and a common weed *Chenopodium album*. Their results showed that each plant harbored distinct rhizosphere bacterial communities despite the fact that both plants were growing in the same soil.

Random Amplified Polymorphic DNA and DNA Amplification Fingerprinting

Random amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF) techniques utilize PCR amplification with a short (usually ten nucleotides)

primer, which anneals randomly at multiple sites on the genomic DNA under low annealing temperature, typically ≤35°C (Franklin et al. 1999). These methods generate PCR amplicons of various lengths in a single reaction that are separated on agarose or polyacrylamide gel depending on the genetic complexity of the microbial communities. Because of the high speed and ease of use, RAPD/DAF has been used extensively in fingerprinting overall microbial community structure and closely related bacterial species and strains (Franklin et al. 1999). Both RAPD and DAF are highly sensitive to experimental conditions (e.g., annealing temperature, $MgCl_2$ concentration) and quality and quantity of template DNA and primers. Thus, several primers and reaction conditions need to be evaluated to compare the relatedness between microbial communities and obtain the most discriminating patterns between species or strains. A RAPD profiling study was used with 14 random primers to assess changes in microbial diversity in soil samples that were treated with pesticides (triazolone) and chemical fertilizers (ammonium bicarbonate) (Yang et al. 2000). RAPD fragment richness data demonstrated that pesticide-treated soil maintained an almost identical level of diversity at the DNA level as the control soil (i.e., without contamination). In contrast, chemical fertilizer caused a decrease in the DNA diversity compared to control soil.

Amplified Ribosomal DNA Restriction Analysis

Amplified ribosomal DNA restriction analysis (ARDRA) is based on DNA sequence variations present in PCR-amplified 16S rRNA genes (Smit et al. 1997). The PCR product amplified from environmental DNA is generally digested with tetracutter restriction endonucleases (e.g., *Alu*I, and *Hae*III), and restricted fragments are resolved on agarose or polyacrylamide gels. Although ARDRA provides little or no information about the type of microorganisms present in the sample, the method is still useful for rapid monitoring of microbial communities over time, or to compare microbial diversity in response to changing environmental conditions. ARDRA is also used for identifying the unique clones and estimating OTUs in environmental clone libraries based on restriction profiles of clones (Smit et al. 1997). One of the major limitations of ARDRA is that restriction profiles generated from complex microbial communities are sometimes too difficult to resolve by agarose/PAGE. The ARDRA technique was applied for assessing the effect of copper contamination on the microbial communities in soil. Whole community ARDRA profiles showed a lower diversity in copper-contaminated soil compared with control soil with no contamination (Smit et al. 1997).

Terminal Restriction Fragment Length Polymorphism

Terminal restriction fragment length polymorphism (T-RFLP) is similar to ARDRA except for one major difference, which is the use of one 5′ fluorescently labeled primer during the PCR reaction. The resulting PCR products are digested with

restriction enzyme(s), and terminal restriction fragments (T-RFs) are separated on an automated DNA sequencer (Thies 2007). Only the terminally fluorescent labeled restriction fragments are detected, thus simplifying the banding pattern and allowing analysis of complex microbial communities. Community diversity is estimated by analyzing the size, numbers, and peak heights of resulting T-RFs. Each T-RF is assumed to represent a single OTU or ribotype. With recent developments in bioinformatics, several Web-based T-RFLP analysis programs have been developed, which enable researchers to rapidly assign putative identities based on a database of fragments produced by known 16S rDNA sequences. Similar to ARDRA, a T-RFLP pattern is characteristic of the restriction enzyme(s) used, and more than two enzymes should typically be applied. One pitfall of T-RFLP method is that it underestimates community diversity because only a limited number of bands per gel (generally <100) can be resolved, and different bacterial species can share the same T-RF length (OTU overlap or OTU homoplasy). Nonetheless, the method does provide a robust index of community diversity, and T-RFLP results are generally very well correlated with the results from clone libraries (Fierer and Jackson 2006). Fierer and Jackson (2006) applied the T-RFLP technique to understand the biogeographical patterns in soil bacterial communities and to investigate the biotic and abiotic factors that shape the composition and diversity of bacterial communities. They collected 98 soil samples from across North and South America representing a wide range of temperature, pH, and other geographical conditions. Their results demonstrated that bacterial diversity was higher in neutral soils compared to acidic soils and was unrelated to factors such as site temperature, latitude, and other variables that typically act as good predictors of animal and plant diversity.

Length Heterogeneity PCR

Length heterogeneity PCR (LH-PCR) analysis is similar to the T-RFLP method except that the latter detects amplicon length variations that are produced after restriction digestion, whereas in LH-PCR different microorganisms are discriminated based on natural length polymorphisms that occur due to mutation within genes (Mills et al. 2007). Amplicon LH-PCR interrogates the hypervariable regions present in 16S rRNA genes and produces a characteristic profile. LH-PCR utilizes a fluorescent dye-labeled forward primer, and a fluorescent internal size standard is run with each sample to measure the amplicon lengths in base pairs. The intensity (height) or area under the peak in the electropherogram is proportional to the relative abundance of that particular amplicon. One advantage of using LH-PCR over the T-RFLP is that the former does not require any restriction digestion and therefore PCR products can be directly analyzed by a fluorescent detector. The limitations of LH-PCR technique include inability to resolve complex amplicon peaks and underestimation of diversity, as phylogenetically distinct taxons may produce same-length amplicons (Mills et al. 2007). LH-PCR was used in combination with fatty acid methyl ester (FAME) analysis to investigate the microbial communities in soil samples that differed in terms of type and/or crop management practices

(Ritchie et al. 2000). LH-PCR results strongly correlated with FAME analysis and were highly reproducible, and successfully discriminated different soil samples. The most abundant bacterial community members, based on cloned LH-PCR products, were members of the β-Proteobacteria, Cytophaga–Flexibacter–Bacteriodes, and the high-G+C-content Gram-positive bacterial group.

Ribosomal Intergenic Spacer Analysis

Ribosomal intergenic spacer analysis (RISA) involves PCR amplification of a portion of the intergenic spacer region (ISR) present between the small (16S) and large (23S) ribosomal subunits (Fisher and Triplett 1999). The ISR contains significant heterogeneity in both length and nucleotide sequence. By using primers annealing to conserved regions in the 16S and 23S rRNA genes, RISA profiles can be generated from most of the dominant bacteria existing in an environmental sample. RISA provides a community-specific profile, with each band corresponding to at least one organism in the original community. The automated version of RISA is known as ARISA and involves use of a fluorescence-labeled forward primer, and ISR fragments are detected automatically by a laser detector. ARISA allows simultaneous analysis of many samples; however, the technique has been shown to overestimate microbial richness and diversity (Fisher and Triplett 1999). Ranjard et al. (2001) evaluated ARISA to characterize the bacterial communities from four types of soil differing in geographic origins, vegetation cover, and physicochemical properties. ARISA profiles generated from these soils were distinct and contained several diagnostic peaks with respect to size and intensity. Their results demonstrated that ARISA is a very effective and sensitive method for detecting differences between complex bacterial communities at various spatial scales (between- and within-site variability).

2.3.1.3 DNA Microarrays

DNA microarrays have been used primarily to provide a high-throughput and comprehensive view of microbial communities in environmental samples. The PCR products amplified from total environmental DNA is directly hybridized to known molecular probes, which are attached on the microarrays (Gentry et al. 2006). After the fluorescently labeled PCR amplicons are hybridized to the probes, positive signals are scored by the use of confocal laser scanning microscopy. The microarray technique allows samples to be rapidly evaluated with replication, which is a significant advantage in microbial community analyses. In general, the hybridization signal intensity on microarrays is directly proportional to the abundance of the target organism. Cross hybridization is a major limitation of microarray technology, particularly when dealing with environmental samples. In addition, the microarray is not useful in identifying and detecting novel prokaryotic taxa. The ecological importance of a genus could be completely ignored if the genus does not have a corresponding probe on the microarray. DNA microarrays used in microbial

ecology could be classified into two major categories depending on the probes: (1) 16S rRNA gene microarrays and (2) functional gene arrays (FGA).

16S rRNA gene Microarrays (PhyloChip)

The universal high-density 16S microarray contains about 30,000 probes of 16S rRNA gene targeted to several cultured microbial species and "candidate divisions" (DeSantis et al. 2007). These probes targets all 121 demarcated prokaryotic orders and allow simultaneous detection of 8,741 bacterial and archaeal taxa. PhyloChip technology has been used for rapid profiling of environmental microbial communities during bioterrorism surveillance, bioremediation, climate change, and source tracking of pathogen contamination (Brodie et al. 2007; DeSantis et al. 2007). PhyloChips were used to investigate the indigenous soil bacterial communities in two abandoned uranium mine sites, the Edgemont and the North Cave Hills in South Dakota (Rastogi et al. 2010). PhyloChip analysis revealed greater diversity than corresponding clone libraries at each taxonomic level and indicated the existence of 1,300–1,700 bacterial species in uranium mine soil samples. Most of these species were members of the phylum Proteobacteria and contained lineages that were capable of performing uranium immobilization and metal reduction.

Functional Gene Arrays

Unlike PhyloChips that are useful in detecting microbial community composition and contain 16S rRNA genes as probes, FGA are designed primarily to detect specific metabolic groups of bacteria. Thus, FGA not only reveal the community structure, but they also shed light on the in situ community metabolic potential. FGA contain probes from genes with known biological functions; therefore, they are also useful in linking microbial community composition to ecosystem functions. For instance, an FGA termed GeoChip contains >24,000 probes from all known metabolic genes involved in various biogeochemical, ecological, and environmental processes such as ammonia oxidation, methane oxidation, and nitrogen fixation (He et al. 2007). GeoChips have been used to interrogate the role of Antarctica soil microbial communities in the global biogeochemical cycling of carbon and nitrogen (Yergeau et al. 2009). Their study demonstrated a significant correlation between the distribution of key genes and soil temperature, chemical characteristics, and vegetation cover. For example, the relative detection of cellulose degradation genes was correlated with temperature, and microbial carbon-fixation genes were found in greater abundance in samples without vegetation.

2.3.1.4 Quantitative PCR

Quantitative PCR (Q-PCR), or real-time PCR, has been used in microbial investigations to measure the abundance and expression of taxonomic and functional gene

markers (Bustin et al. 2005; Smith and Osborn 2009). Unlike traditional PCR, which relies on end-point detection of amplified genes, Q-PCR uses either intercalating fluorescent dyes such as SYBR Green or fluorescent probes (TaqMan) to measure the accumulation of amplicons in real time during each cycle of the PCR. Software records the increase in amplicon concentration during the early exponential phase of amplification which enables the quantification of genes (or transcripts) when they are proportional to the starting template concentration. When Q-PCR is coupled with a preceding reverse transcription (RT) reaction, it can be used to quantify gene expression (RT-Q-PCR). Q-PCR is highly sensitive to starting template concentration and measures template abundance in a large dynamic range of around six orders of magnitude. Several sets of 16S and 5.8S rRNA gene primers have been designed for rapid Q-PCR based quantification of soil bacterial and fungal microbial communities (Fierer et al. 2005). Q-PCR has also been successfully used in environmental samples for quantitative detection of important physiological groups of bacteria such as ammonia oxidizers, methane oxidizers, and sulfate reducers by targeting *amoA*, *pmoA*, and *dsrA* genes, respectively (Foti et al. 2007). Kolb et al. (2003) estimated the abundance of total methanotrophic population and specific groups of methanotrophs in a flooded rice field soil by Q-PCR assay of the *pmoA* genes. The total population of methanotrophs was found to be 5×10^6 *pmoA* molecules g^{-1}, and *Methylosinus* (2.7×10^6 *pmoA* molecules g^{-1}) and *Methylobacter/Methylosarcina* groups (2.0×10^6 *pmoA* molecules g^{-1}) were the dominant methanotrophs. The *Methylocapsa* group was below the detection limit of Q-PCR (1.9×10^4 *pmoA* molecules g^{-1}).

2.3.1.5 Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) enables in situ phylogenetic identification and enumeration of individual microbial cells by whole cell hybridization with oligonucleotide probes (Amann et al. 1995). A large number of molecular probes targeting 16S rRNA genes have been reported at various taxonomic levels (Amann et al. 1995). The FISH probes are generally 18–30 nucleotides long and contain a fluorescent dye at the 5′ end that allows detection of probe bound to cellular rRNA by epifluorescence microscopy. In addition, the intensity of fluorescent signals is correlated to cellular rRNA contents and growth rates, which provide insight into the metabolic state of the cells. FISH can be combined with flow cytometry for a high-resolution automated analysis of mixed microbial populations. The FISH method was used to follow the dynamics of bacterial populations in agricultural soils treated with s-triazine herbicides (Caracciolo et al. 2010). A variety of molecular probes were used to target specific phylogenetic groups of bacteria such as α , β , γ , and δ subdivisions of Proteobacteria and Planctomycetes. Results demonstrated that γ-Proteobacteria populations diminished sharply after 14 days of incubation in treated soil compared to control soil with no s-triazine treatment. In contrast, β-Proteobacteria populations remained higher than that of the control soils throughout the incubation period (70 days). Other bacterial groups, e.g., α-Proteobacteria and Planctomycetes were not significantly affected by the presence of the herbicide.

Low signal intensity, background fluorescence, and target inaccessibility are commonly encountered problems in FISH analysis. In the last few years, extensive improvements have been made to solve some of these problems which include the use of brighter fluorochromes, chloramphenicol treatment to increase the rRNA content of active bacterial cells, hybridization with probes carrying multiple fluorochromes, and signal amplification with reporter enzymes (Rogers et al. 2007). In a modified FISH method known as *ca*talyzed *r*eporter *d*eposition (CARD) FISH, the hybridization signal is enhanced through the use of tyramide-labeled fluorochromes (Pernthaler et al. 2002). This allows the accumulation of several fluorescent probes at the target site, which ultimately increases the signal intensity and sensitivity. Li et al. (2008) developed an advanced imaging technique by combining FISH to secondary-ion mass spectrometry (SIMS). In principle, the technique uses 16S rRNA probes for in situ hybridization; however, the probes are labeled with a stable isotope or element (e.g., fluorine or bromine atoms) rarely present in biomass. Once the probe is hybridized, the microbial identities of stable isotope-labeled cells are simultaneously determined in situ by NanoSIMS imaging. With next-generation SIMS instruments, spatial resolution of ~50 nm (NanoSIMS) was achieved, which allowed quantifying the isotopic composition at single-cell level.

2.3.1.6 Microbial Lipid Analysis

Microbial community characterization by biomolecules other than nucleic acids such as lipids has been used without relying on culturing (Banowetz et al. 2006). Fatty acids are present in a relatively constant proportion of the cell biomass, and signature fatty acids exist in microbial cells that can differentiate major taxonomic groups within a community. The fatty acids are extracted by saponification followed by derivatization to give the respective FAMEs, which are then analyzed by gas chromatography. The emerging pattern is then compared to a reference FAME database to identify the fatty acids and their corresponding microbial signatures by multivariate statistical analyses. FAME profiling and multivariate statistical methods were used to identify the sources of soil that were contaminating surface waters (Banowetz et al. 2006). A variety of reference soils collected from land with contrasting uses in different seasons was used to generate FAME fingerprints for reliable classification of soils. FAME fingerprints generated from different soil samples were capable of discriminating reference soils. Results showed that FAME analysis can successfully classify sediment samples provided soil FAME profiles are developed for reference soils collected at the same time as surface water samples.

2.3.2 Whole Community Analysis Approaches

Sequence analysis of 16S rRNA genes is commonly used in most microbial ecological surveys. However, being a highly conserved molecule, the 16S rRNA gene does not provide sufficient resolution at species and strain level (Konstantinidis

et al. 2006). Whole-genome molecular techniques offer a more comprehensive view of genetic diversity compared to PCR-based molecular approaches that target only a single or few genes. These techniques attempt to analyze all the genetic information present in total DNA extracted from an environmental sample or pure culture.

2.3.2.1 DNA–DNA Hybridization Kinetics

Whole-genome DNA–DNA hybridization (DDH) offers true genome-wide comparison between organisms. A value of 70% DDH was proposed as a recommended standard for bacterial species delineation (Goris et al. 2007). Typically, bacterial species having 70% or greater genomic DNA similarities usually have >97% 16S rRNA gene sequence identity. Although DDH techniques have been originally developed for pure culture comparisons, they have been modified for use in whole microbial community analysis. In DDH technique, total community DNA extracted from an environmental sample is denatured and then incubated under conditions that allow them to hybridize or reassociate. The rate of DNA reassociation is correlated with the genomic complexity (diversity) present in the sample. If the sample has high sequence diversity, the rate of DNA reassociation will decrease. Under defined conditions, the time needed for half of the DNA to reassociate (the half association value C_0t, where C_0 is the concentration of single-stranded DNA at time zero and t is time) is proportional to genomic diversity and can be used as a diversity index. Based on DDH data, 6,000–10,000 different prokaryotic genomes per gram of soil have been suggested (Torsvik and Øvreås 2002). This number could be much higher as genomes representing rare and unrecovered species might have been overlooked in the analysis.

2.3.2.2 Guanine-Plus-Cytosine Content Fractionation

Different prokaryotic groups differ in their guanine-plus-cytosine (G+C) content of DNA, and phylogenetically related bacterial groups only vary by 3–5% in their G+C content (Nüsslein and Tiedje 1999). Thus, the fractionation of total community DNA can be achieved by density-gradient centrifugation based on G+C content. The technique generates a fractionated profile of the entire community DNA and indicates relative abundance of DNA (hence taxa) as a function of G+C content. The total community DNA is physically separated into highly purified fractions, each representing a different G+C content that can be analyzed by additional molecular techniques such as DGGE/ARDRA to better assess total community diversity. However, G+C content fractionation technique provides a coarse level of phylogenetic resolution as different phylogenetic groups may have the same G+C range. Additionally, it requires a large amount of DNA (about 50 μg) and a total time of about 4 days for completion. G+C fractionation has been widely applied in investigation of soil microbial communities to evaluate the effect of different treatments or management practices (e.g., change in vegetation, grazing, application of

pesticides, and compost application). Nüsslein and Tiedje (1999) applied G+C fractionation together with ARDRA and 16S rRNA gene sequence analyses to investigate the influence of forest versus pasture vegetation in Hawaiian soil microbial communities. All three techniques demonstrated that plants are an important determinant of microbial community structure and shift in vegetative cover to pasture resulted in about 50% change in the microbial community composition.

2.3.2.3 Whole-Microbial-Genome Sequencing

Exploring microbial systems through whole-genome analysis is a comprehensive and integrated approach to understand microbial ecology and function. Whole microbial genomes are sequenced using a shotgun cloning method that involves (1) extraction of DNA from pure cultures, (2) random fragmentation of obtained genomic DNA into small fragments of ~2 kb, (3) ligation and cloning of DNA fragments into plasmid vectors, and (4) bidirectional sequencing of DNA fragments. Once the sequences are obtained, they are aligned and assembled into finished sequences using specialized computer programs such as MEGAN (*ME*ta*G*enome *AN*alyzer) (Huson et al. 2007). The sequences are annotated in open reading frames (ORFs) to predict the encoded proteins (functions). Whole-genome sequencing has provided unprecedented insights into microbial processes at the molecular level and has potential applications in individual and community ecology, bioenergy production, bioremediation, human and plant health, and various industries (Ikeda et al. 2003). Several institutions and laboratories such as The Institute of Genome Research, the U.S. Department of Energy's Joint Genome Institute, Lawrence Berkeley National Laboratory, and J. Craig Venter Institute have completed sequencing of whole genomes of several important microorganisms such as *Pseudomonas syringae* DC3000 (a plant pathogen), *Desulfovibrio desulfuricans* G20 (bioremediation capabilities), and *Methanosaeta thermophila* (a thermophilic aceticlastic methanogen). The genome sequence of *Desulfovibrio desulfuricans* G20, a model sulfate-reducing δ-proteobacterium, demonstrated the existence of metabolic pathways by which these bacteria are able to reduce toxic metals such as uranium(VI) and chromium(VI) to less water-soluble species (Li et al. 2009). These molecular insights were highly crucial for the use of sulfate-reducing bacteria in bioremediation of metal-contaminated groundwater or soils. Recent developments in short-read sequencing techniques such as pyrosequencing have dramatically reduced the time and cost needed for whole-microbial-genome sequencing projects (Metzker 2010). The enormous amount of data gathered from genome sequencing programs is deposited in searchable databases that could be mined with various powerful bioinformatic tools available at the Integrated Microbial Genomes (IMG) Web server (Markowitz et al. 2010) for evolutionary studies, comparative genomics, and proteomics. For example, Microbial Genomes Resources at the National Center for Biotechnology Information (NCBI) is a public database for prokaryotic genome sequencing projects and has now 1,000 complete prokaryotic genomes (http://www.ncbi.nlm.nih.gov/genomes/ [verified on 15th May, 2010]). The Genomes

Online Database (GOLD) is another database resource for comprehensive information regarding complete and ongoing genome projects, as well as metagenomes and metadata, around the world (http://www.genomesonline.org). As of 15th May, 2010, the GOLD database held 1,284 completed and published genomes and 4,289 ongoing bacterial, 199 archaeal, and 1,338 eukaryotic sequencing projects.

2.3.2.4 Metagenomics

Metagenomics is the investigation of collective microbial genomes retrieved directly from environmental samples and does not rely on cultivation or prior knowledge of the microbial communities (Riesenfeld et al. 2004). Metagenomics is also known by other names such as environmental genomics or community genomics, or microbial ecogenomics. Essentially, metagenomics does not include methods that interrogate only PCR-amplified selected genes (e.g., genetic fingerprinting techniques) as they do not provide information on genetic diversity beyond the genes that are being amplified. In principle, metagenomic techniques are based on the concept that the entire genetic composition of environmental microbial communities could be sequenced and analyzed in the same way as sequencing a whole genome of a pure bacterial culture as discussed in the preceding section. Metagenomic investigations have been conducted in several environments such as soil, the phyllosphere, the ocean, and acid mine drainage and have provided access to phylogenetic and functional diversity of uncultured microorganisms (Handelsman 2004). Thus, metagenomics is crucial for understanding the biochemical roles of uncultured microorganisms and their interaction with other biotic and abiotic factors. Environmental metagenomic libraries have proved to be great resources for new microbial enzymes and antibiotics with potential applications in biotechnology, medicine, and industry (Riesenfeld et al. 2004; Rondon et al. 2000). Metagenomic library construction involves the following steps: (1) isolation of total DNA from an environmental sample, (2) shotgun cloning of random DNA fragments into a suitable vector, and (3) transforming the clones into a host bacterium and screening for positive clones. Metagenomic libraries containing small DNA fragments in the range of 2–3 kb provide better coverage of the metagenome of an environment than those with larger fragments. It has been estimated that to retrieve the genomes from rare members of microbial communities at least 10^{11} genomic clones would be required (Riesenfeld et al. 2004). Small-insert DNA libraries are also useful to screen for phenotypes that are encoded by single genes and for reconstructing the metagenomes for genotypic analysis. Large-fragment metagenomic libraries (100–200 kb) are desirable while investigating multigene biochemical pathways. Metagenomic libraries could be screened either by sequence-driven metagenomic analysis that involves massive high-throughput sequencing or by functional screening of expressed phenotypes. Sequence-driven massive whole-genome metagenomic sequencing sheds light on many important genomic features such as redundancy of functions in a community, genomic organizations, and traits that are acquired from distinctly related taxa through horizontal gene transfers (Handelsman 2004).

In function-driven metagenomic analysis (functional metagenomics), libraries are screened based on the expression of a selected phenotype on a specific medium. A wide variety of biochemical activities have been discovered in environmental metagenomic libraries. For example, novel antibiotics (e.g., turbomycin, terragine), microbial enzymes (e.g., cellulases, lipases, amylases), and proteins (e.g., antiporters) have been identified in soil metagenomic libraries (Rondon et al. 2000). Function-driven metagenomic approaches require successful expression of a desired gene in a heterologous host such as *E. coli*. Thus, a major limitation is very low level or no expression of the majority of environmental genes in *E. coli*. In some cases, improved gene expression can be achieved by transforming metagenomic DNA into several additional surrogate hosts such as *Streptomyces*, *Bacillus*, *Pseudomonas*, and *Agrobacterium*. Strategies that can enhance heterologous expression of unknown genes in host cells are highly desirable. For example, genetically engineered *E. coli* that can support the translation and transcription of wide diversity of genes, or cloning vectors with strong promoters that can provide additional transcription factors will be highly desirable. In a metagenomic library, the frequency of active gene clones expressing a phenotype is typically very low. For example, in an environmental metagenomic library established from soil, only one in 730,000 clones showed lipolytic activity (Henne et al. 2000). The DNA and inferred protein sequence of a novel lipolytic clone exhibited only a moderate identity (<50%) with known lipases, indicating that it could be from an uncultured organism. Low occurrence of actively expressing clones in metagenomic libraries necessitates improved high-throughput screening and detection assays.

2.4 Next-Generation DNA Sequencing Techniques Transform Microbial Ecology

Large-scale sequencing technologies allow us to investigate deeper and deeper layers of the microbial communities and are vital in presenting an unbiased view of phylogenetic composition and functional diversity of environmental microbial communities (Zwolinski 2007). The capability of large-scale sequencing techniques to generate billions of reads at low cost with high speed is useful in many applications such as whole-genome sequencing, metagenomics, metatranscriptomics, and proteogenomics. Recent developments in new sequencing chemistries, bioinformatics, and instruments have revolutionized the field of microbial ecology and genomics. Next-generation sequencing platforms such as Roche/454, Illumina/Solexa, Life/APG, and HeliScope/Helicos BioSciences are much faster and less expensive than traditional Sanger's dideoxy sequencing of cloned amplicons (Metzker 2010). 454Life Sciences commercially developed a 454 pyrosequencing technique, which allows massive parallel high-throughput sequencing of hypervariable regions of 16S rRNA genes and offers two to three orders of magnitude higher coverage of microbial diversity than typical Sanger sequencing of a few hundred 16S rRNA gene clones. The hypervariable regions

targeted are short enough (100–350 bases) but provide sufficient phylogenetic information and are easily covered in the short read lengths generated by pyrosequencing techniques.

One advantage of using the pyrosequencing technique is that multiple environmental samples can be combined in a single run, and after sequencing, the reads can be parsed through their assigned nucleotide barcode, which is added in templates during PCR. The latest release of the third-generation platform 454 Genome Sequencer XLR (GS FLX Titanium) can yield read lengths exceeding >450 bp and approximately 400 million high-quality bases per 10-h instrument run with an accuracy of 99.96% (Metzker 2010). Third-generation sequencing platforms developed by Helicos and Pacific Biosciences are expected to be released in the year 2010 and would be capable of single-molecule sequencing and producing reads exceeding more than 1 kb with an accuracy of >99.99% (Metzker 2010).

Environmental samples such as soil contain huge genetic diversity that encompasses microorganisms from the Eukarya, Bacteria, and Archaea domains. For example, GenBank, the largest database of microbial sequences, provides >686,266 sequence entries when searched for the keyword "soil" (verified on 15 May 2010). This vast genetic information available in databases is the evidence of advances in genomics and increased use of nucleic-acid sequencing. Until recently, first-generation automated Sanger sequencing has been used in most molecular microbial surveys. The major limiting factor in the Sanger technique was the cost and time involved, with the result that most of the studies included sequencing of only few hundred clones. Sequencing of a low number of clones captures only the dominant components of microbial communities that mask the detection of low-abundance microorganisms. These low-abundance microorganisms constitute a highly diverse "rare biosphere" in almost every environmental sample including soil (Lauber et al. 2009). The rare biosphere microbial populations are largely unexplored and offer a potentially inexhaustible genetic reservoir that could be explored only by using next-generation sequencing techniques. In a molecular investigation, spatial changes in soil bacterial communities were explored by targeting V1 and V2 hypervariable regions of 16S rRNA genes using a massive barcoded pyrosequencing technique (Lauber et al. 2009). Eighty-eight soil samples representing a wide range of ecosystems from across North and South America were collected, and a total of 152,359 high-quality sequences on average of 1,501 sequences per sample were generated. Results suggested enormous phylogenetic diversity in soil microbial communities with an average of at least 1,000 species per soil sample. The dominant phyla in all soil samples were Acidobacteria, Alphaproteobacteria, Actinobacteria, Bacteroidetes, and Beta/Gammaproteobacteria. The Lauber et al. (2009) study demonstrated that even after sequencing more than 1.5 billion 16S rRNA gene amplicons, the full extent of species diversity was not covered. This provided further evidence that soil bacterial communities are extremely diverse and contain a large "rare biosphere" represented by an enormous number of low-abundance unique taxa. Such studies highlight the importance of large-scale sequencing techniques in investigating the highly diverse soil microbial communities.

2.5 Functional Microbial Ecology: Linking Community Structure and Function

Understanding how microbial communities function in natural environments is a central goal in microbial ecology. RNA extracted from environmental samples provides more valuable information than DNA in revealing active microbial communities versus dormant microbial communities (Torsvik and Øvreås 2002). This is due to the fact that rRNA and mRNA are considered as indicators of functionally active microbial populations. The amount of rRNA in a cell roughly correlates with the growth activity of bacteria, and mRNA of functional genes allows the detection and identification of bacteria actually expressing key enzyme activities under specific conditions (Wellington et al. 2003). Several genes, e.g., *amoA* (ammonia oxidation), *nifH* (nitrogen fixation), *nirK* and *nirS* (denitrification), and *dsrA* (sulfate reduction), have been amplified from DNA/RNA isolated from microbial communities to obtain insights into key microbial processes (Hansel et al. 2008). Microbial catabolic diversity could also be studied by enzyme-coding genes involved in utilization of specific carbon substrates such as chitin, cellulose, and lipids (Torsvik and Øvreås 2002). The diversity of lipase-producing microorganisms in glacier soil was investigated by the PCR amplification of lipase genes, and sequence analysis showed the existence of several novel lipase-producing organisms in soil (Yuhong et al. 2009). More advanced methods utilizing stable isotopes such as stable isotope probing (SIP), microautoradiography–FISH (MAR–FISH), and Raman–FISH offer more detailed insights into the metabolic activities of microbial communities and are discussed in the following sections.

2.5.1 Stable Isotope Probing

SIP involves offering a stable isotope (e.g., ^{13}C)-labeled substrate to microbial communities whose utilization is of interest to decipher a key biogeochemical process (Wellington et al. 2003). Active microbial communities that utilize the labeled substrate during growth incorporate the isotopes within their biomass. The labeled biomolecules (e.g., DNA, RNA, phospholipid fatty acids [PLFA]) are then separated from biomass by different biochemical methods, and the phylogenetic identity of microorganisms metabolizing the substrate is established using molecular techniques. SIP relying on DNA biomarkers involves labeling of DNA with ^{13}C that could be separated from ^{12}C by CsCl equilibrium density-gradient centrifugation. The ^{13}C-labeled DNA could be analyzed by genetic fingerprinting or clone library techniques, leading to the identification of microorganisms. SIP was applied to decipher the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading soil microbial communities (Cupples and Sims 2007). Soil samples were amended with ^{13}C-labeled 2,4-D and were incubated for 17 days. After incubation, labeled DNA was purified from soil samples and was used to construct 16S rRNA clone libraries.

Phylogenetic analyses of clone sequences revealed that bacteria belonging to β-Proteobacteria such as Comamonadaceae and *Ramlibacter* were responsible for uptake and degradation of the herbicide.

In recent years, with advances in imaging and spectroscopic techniques, SIP has been combined with other techniques such as FISH and Raman microscopy to simultaneously investigate the taxonomic identities and activity of microbial communities at single-cell resolution (Huang et al. 2007). In the Raman–FISH method, environmental samples are incubated with a substrate labeled with ^{13}C stable isotope. After incorporation, the spectral profiles of uncultured microbial cells at single-cell resolution are generated using Raman microscopy, which measures the laser light scattered by chemical bonds of different cell biomarkers. The proportion of stable isotope incorporation in cells affects the amount of light scattered, resulting in measurable peak shifts for labeled cellular components. The Raman–FISH provides much higher resolution and overcomes many of the limitations associated with conventional SIP/MAR–FISH techniques. Huang et al. (2007) used the Raman–FISH method to investigate naphthalene-degrading *Pseudomonas* communities in groundwater. Their results, based on differences in ^{13}C content of the various pseudomonad cells, suggested that different *Pseudomonas* species and even members of the same species vary in their capability of naphthalene degradation.

2.5.2 Microautoradiography

Microautoradiography (MAR) relies on the fact that metabolically active cells utilizing radiolabeled substrate can be visualized by exposure to radiation-sensitive silver halide emulsion (Okabe et al. 2004). The emulsion is placed on the top of cells that are mounted on a microscope slide. After exposure, excited silver ions precipitate as black grains of metallic silver inside or adjacent to the cells that can be observed by transmission electron microscopy. Commonly used radiolabeled substrates include glucose, acetate, and amino acids, which provide a general view of the overall metabolic diversity. More specific substrates along with selective growth (incubation) conditions have been used to identify important physiological processes in situ. For example, radiolabeled iron or sulfate can be provided under controlled anaerobic conditions to identify the iron- and sulfate-reducing microbial communities, respectively. When MAR is used in combination with FISH (MAR–FISH), it allows simultaneous phylogenetic identification of active cells that consume the radioactive substrate (Rogers et al. 2007). MAR–FISH has been modified slightly, leading to other methods such as STAR (*s*ubstrate *t*racking *a*uto*r*adiography)–FISH. However, STAR–FISH differs from MAR–FISH only in methodological details, and the basic principle of the technique remains the same. Nielsen et al. (2003) developed a quantitative MAR (QMAR)–FISH approach that can detect even single cells due to its improved fixation protocol and use of an internal standard of bacteria with known specific radioactivity. MAR–FISH technique was used to study the autotrophic nitrifying bacteria in biofilms (Okabe et al. 2005). The uptake by heterotrophic bacteria

of ^{14}C-labeled products derived from nitrifying bacteria was directly visualized by MAR–FISH. Results revealed that members belonging to Chloroflexi and Cytophaga–Flavobacterium play an important role in scavenging the dead biomass and metabolites of nitrifying bacteria and ultimately preventing the accumulation of organic waste products in the biofilms.

2.5.3 Isotope Array

Isotope arrays allow for functional and phylogenetic screening of active microbial communities in a high-throughput fashion. The technique uses a combination of SIP for monitoring the substrate uptake profiles and microarray technology for deciphering the taxonomic identities of active microbial communities (Adamczyk et al. 2003). In principle, samples are incubated with a ^{14}C-labeled substrate, which during the course of growth becomes incorporated into microbial biomass. The ^{14}C-labeled rRNA is separated from unlabeled rRNA and then labeled with fluorochromes. Fluorescent labeled rRNA is hybridized to a phylogenetic microarray followed by scanning for radioactive and fluorescent signals. The technique thus allows parallel study of microbial community composition and specific substrate consumption by metabolically active microorganisms of complex microbial communities. The major strengths of the technique lie in the fact that it does not involve any amplification step and is hence free of biases associated with PCR. The limitations of the technique include difficulties in obtaining high-quality rRNA and detecting low abundance but active microbial populations from environmental samples (Adamczyk et al. 2003). Adamczyk et al. (2003) successfully used this technique to demonstrate phylogenetic diversity and CO_2 fixation activity of ammonia-oxidizing bacteria (AOB) in nitrifying activated sludge samples. Their results suggested that *Nitrosomonas* was the dominant lineage in AOB communities of sludge samples.

2.6 Postgenomic Approaches

The recent applications of DNA-based molecular techniques such as metagenomics have revealed new insights into the phylogenetic and functional diversity of microbial communities. However, in the postgenomic era, the limitations of DNA-based molecular approaches have been realized. For example, DNA-based techniques do not provide information on the gene expression (functionality) as it occurs under in situ conditions (Wilmes and Bond 2006). With the availability of comprehensive metagenomic databases, which also includes genomic sequences from uncultured microorganisms, it is now possible to apply postgenomic approaches such as metaproteomics and metatranscriptomics to reveal the link between genetic potential and functionality in microbial communities. In the following sections, these techniques are discussed in detail with their potential applications in investigating functionality of microbial communities.

2.6.1 Metaproteomics

Metaproteomics, also commonly known as environmental proteomics, deals with the large-scale study of proteins expressed by environmental microbial communities at a given point in time (Wilmes and Bond 2006; Keller and Hettich 2009). Compared to other cell molecules such as lipids and nucleic acids, protein biomarkers are more reliable and provide a clearer picture of metabolic functions than functional genes or even the corresponding mRNA transcripts of microbial communities (Wilmes and Bond 2006). Although methods such as SIP/MAR–FISH have been developed for structure–function analyses of microbial communities, these methods reveal information only on microbial communities associated with a specific biogeochemical process (e.g., nitrification, methane oxidation) and do not reveal an overall picture of microbial functionalities. Compared to these methods, proteomics offers a comprehensive approach to investigate the physiology of microbial communities both qualitatively and quantitatively. For example, proteomic profiling of microbial communities provides critical information on protein abundances and protein–protein interactions, which could not be achieved by DNA/RNA molecular techniques such as metatranscriptomics and metagenomics (Keller and Hettich 2009). The physiological responses of microbial communities due to a stress condition could be identified from an altered proteofingerprint, which reflects changes in the functional status of the communities. Once the proteins are identified, they could be linked to corresponding metagenomic sequences to link metabolic functions to individual microbial species.

Methodologically, metaproteome analysis involves extraction of total proteins from an environmental sample. Although in situ protein lysis methods provide an exhaustive recovery, a significant amount of protein originates from other organisms such as protozoa, fungi, and multicellular organisms, which further complicate the taxonomic characterization of proteins (Keller and Hettich 2009). Therefore, in some cases, microbial cells are first separated from the environmental matrix by ultracentrifugation and then lysed, which allows obtaining much higher quality and quantity of bacterial proteins. Once the total protein is obtained, it is separated by one-dimensional and two-dimensional electrophoresis to generate a community proteofingerprint. After separation, protein spots are digested and are identified by a variety of powerful analytical methods. Currently, high-throughput proteomic profiling of microbial communities is possible due to development of chromatographic and mass spectroscopic techniques (MS-based proteomics). High-efficiency mass spectrometry integrated with liquid chromatography allows a highly sensitive and rapid identification of proteins. The availability of Web-based services such as ExPASy (*E*xpert *P*rotein *A*nalysis *S*ystem; http://www.expasy.org/) offers a comprehensive suite of tools that are vital in identification and characterization of protein mass fingerprinting data. A metaproteomic approach was employed to identify proteins that were involved in the biodegradation of chlorophenoxy acid in soil samples (Benndorf et al. 2007). Soil samples were first enriched for chlorophenoxy acid-degrading bacteria by incubating with 2,4-D for a

period of 22 days. After incubation, protein extracts were isolated from soil and separated by SDS-PAGE, and protein bands were identified by liquid chromatography linked to mass spectroscopy. Proteomic analysis identified a major catabolic enzyme 2,4-dichlorophenoxyacetate dioxygenase, membrane transport proteins (porins), and molecular chaperones.

2.6.2 Proteogenomics

In metaproteomics, protein sequences could be identified with confidence only if they have significant homology to existing proteins in available databases. However, in most of the environmental proteomic surveys, proteins are only distantly related to known database sequences. Therefore, it appears that the majority of short protein sequences retrieved from metaproteomes will remain unidentified and cannot be assigned to their functional and phylogenetic features. However, these limitations have been overcome by combining the metaproteomic and metagenomic approaches together under the name of "proteogenomics" (Banfield et al. 2005). In community proteogenomics, total DNA and proteins are extracted from the same sample, which allows linking of biological functions to phylogenetic identity with greater confidence. The metagenomic part of the proteogenomic approach plays a very significant role and increases the identification of protein sequences by metagenomic analysis of the same sample from which the proteins were extracted. The proteogenomics approach was applied to decipher phyllosphere bacterial communities in a study by Delmotte et al. (2009). Bacterial biomass was harvested from leaf surfaces of soybean, clover, and *Arabidopsis*, and proteins were extracted. This was followed by tryptic digestion and separation of fragments by liquid chromatography and analysis by mass spectrometry. This led to the identification of 2,883 unique proteins from nearly one-half million spectra. The metagenomic data generated from the DNA extracted from the same pool of bacterial biomass significantly increased (up to 74%) the number of identified proteins, indicating that the majority of the bacterial communities present in the phyllosphere were genetically distinct from those currently available in databases. Most identified proteins in the phyllosphere proteome were assigned to the three bacterial genera *Methylobacterium*, *Sphingomonas*, and *Pseudomonas*. Large numbers of proteins involved in methanol oxidation were identified and were assigned to *Methylobacterium* species that can use methanol as a source of carbon and energy.

2.6.3 Metatranscriptomics

Metatranscriptomics (or environmental transcriptomics) allows monitoring of microbial gene expression profiles in natural environments by studying global transcription of genes by random sequencing of mRNA transcripts pooled from

microbial communities at a particular time and place (Moran 2009). Metatranscriptomics is particularly suitable for measuring changes in gene expression and their regulation with respect to changing environmental conditions. The major challenge in metatranscriptomics is the fact that prokaryotic microbial mRNA transcripts are not polyA tailed, so obtaining complementary DNA (cDNA) is not easy. This results in coextraction of more abundant rRNA molecules in the total RNA pool, which can lead to overwhelming background sequences in a large-scale sequencing analysis. A method for selectively enriching mRNA by subtractive hybridization of rRNA has been developed and evaluated for the gene transcript analysis of marine and freshwater bacterioplankton communities, which revealed the presence of many transcripts that were linked to biogeochemical processes such as sulfur oxidation (*soxA*), assimilation of C1 compounds (*fdh1B*), and acquisition of nitrogen via polyamine degradation (*aphA*) (Poretsky et al. 2005). More recently, a "double-RNA" method has been devised to analyze the total RNA pool of a community, as it is naturally rich in not only functionally but also taxonomically relevant molecules, i.e., mRNA and rRNA, respectively (Urich et al. 2008). This offers a means to investigate both structural and biochemical activity of microbes in a single experiment. Their study combined transcriptomic profiling with massive pyrosequencing techniques to produce 193,219 rRNA tags and 21,133 mRNA-tags from sandy soil samples that were poor in nutrients and neutral in pH. The rRNA tags provided data on the phylogenetic composition of soil microbial communities and showed that Actinobacteria and Proteobacteria were most abundant, while Crenarchaeota were less abundant in soil samples. The mRNA tags provided a glimpse of the in situ expression of several key metabolic enzymes such as ammonia monooxygenase (*amoA* and *amoC*) and nitrite reductase (*nirk*) that were involved in ammonia oxidation. In addition, microbial gene transcripts coding for the enzymes methyl-malonyl-CoA mutase and 4-hydroxybutyryl-CoA dehydratase that play a role in CO_2 fixation pathways in Crenarchaeaota were detected.

2.7 Bias in Molecular Community Analysis Methods

Like culture methods, molecular techniques have their own pitfalls and are associated with bias at every step (von Wintzingerode et al. 1997). Biases associated with DNA extraction such as incomplete or preferential lysis of certain microbial cells can distort the community composition, richness, and microbial community structure. Feinstein et al. (2009) suggested the use of several validated DNA extraction methods and pooled DNA extracts in PCR-based molecular methods to minimize any risk of bias. Biases associated with PCR could include inhibition by compounds such as humic acids, which are generally coextracted with DNA extracted from soil. Several DNA purification steps have been devised; however, they lead to loss of DNA during purification, which also causes bias in subsequent PCR. Dilution of DNA templates or dialysis can be applied, but it influences the PCR efficiency. Hybridization efficiency and specificity of primers sometimes cause preferential amplification of certain templates, which affects the quantitative assessment of

microbial diversity. Formations of PCR artifacts (e.g., chimeric molecules, deletion mutants, and point mutants) could also lead to misleading results (von Wintzingerode et al. 1997).

2.8 Concluding Remarks and Future Directions

With the development and application of molecular genomic tools, the field of microbial ecology is undergoing unprecedented changes. Postgenomic molecular approaches enable us to interrogate the structural and functional diversity of environmental microbial communities and reveal that we have only scratched the surface of the genetic and metabolic diversity present in the most abundant organisms of the Earth, the *Prokaryotes*. Several important questions such as "How many microbial species are there on the Earth?", "What is the extent of metabolic diversity in natural microbial communities?", and "How microbial communities are governed by biological, chemical and physical factors?" remain to be understood. Understanding the functional roles of uncultured organisms still remains a daunting task, as most of the genes identified have no homologous representatives in databases. Although considerable progress has been made in the characterization of microbial communities by the application of metagenomic, metatranscriptomic, and proteogenomic approaches, many technical challenges remain including DNA, RNA, and protein extraction from environmental samples, mRNA instability, and low abundance of certain gene transcripts in total RNA. The next-generation sequencing techniques are still developing, and many technological innovations particularly tuned for environmental samples are expected in these techniques. Development in bioinformatics tools is also needed for evaluating the tremendous amount of information generated through whole-genome analysis and metagenomic and metatranscriptomics approaches. Quantitative assessment of microbial communities is the greatest challenge due to significant biases associated with nucleic acid isolation and PCR and requires more advanced DNA/RNA extraction techniques for environmental samples. All of the molecular approaches available for community structure and function analysis have advantages and limitations associated with them, and none provides complete access to the genetic and functional diversity of complex microbial communities. A combination of several techniques should be applied to interrogate the diversity, function, and ecology of microorganisms. Culture-based and culture-independent molecular techniques are neither contradictory nor excluding and should be considered as complementary. An interdisciplinary systems approach embracing several "omics" technologies to reveal the interactions between genes, proteins, and environmental factors will be needed to provide new insights into environmental microbiology. Development of multi-"omics" approaches will be a high-priority area of research in the coming years.

Acknowledgement The authors would like to sincerely thank Dr. David E. Cummings, Point Loma Nazarene University, San Diego, CA for providing critical comments and suggestions on this chapter.

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Chapter 3
The Biofilm Returns: Microbial Life at the Interface

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Abstract Scientific studies over the past few decades have shown that the vast majority of microbes in the aqueous environment do not live as free-floating (i.e., planktonic) forms, but rather prefer to live as attached communities termed biofilms. Biofilm formation onto surfaces is usually detrimental to human health and man-made structures; biofilm-related problems range from antibiotic-resistant infections in humans and animals to drinking water contamination, energy loss in industrial systems, and increased corrosion in ship hulls and offshore structures. Biofilms also play several beneficial roles, such as nutrient transformation in the plant rhizosphere and enhanced biodegradation of organic carbon and various pollutants during wastewater treatment and soil bioremediation. Recently, biofilms have shown great potential in selective, low-cost catalysis and energy conversion processes in biofuel production and microbially driven batteries. The biofilm structure provides several advantages to microorganisms within the biofilm, including resistance to biocides and antibiotics, viscoelasticity, and resistance against fluid-dynamic shear stress. The congregation of multiple species into biofilm microcosms increases the range of substrates that can be biodegraded and offers great flexibility for a number of biotechnological applications. In the last 20 years, researchers have unveiled the relationship between biofilm structure and activity and have devised many methods to control biofilm development. However, use of biofilms for contaminant degradation in the field is still in its infancy. Furthermore, the processes that employ biofilms for energy conversion, environmental sensing, and "white biotechnology" (commonly known as industrial biotechnology) are still largely confined to academic research. In this chapter, we aim to highlight the most important and recent advances in the field of biofilm-based technologies and their potential applications.

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_3,

3.1 Introduction

Until recently, most microbiologists have focused on free-floating bacteria grown in defined media. This culturing method has been helpful in the study of microbial pathogenesis and physiology; however, bacteria rarely exist in nature as pure culture planktonic growth. Direct observation of a wide variety of natural habitats has established that the majority of microbes exist within well-structured communities attached onto surfaces and not as free-floating organisms. These attached communities are known as biofilms (Costerton et al. 1999).

Biofilms are important clinically as well as industrially. Clinically, biofilms are the source of persistent infections such as dental caries, nosocomial infections, as well as a variety of other infections and diseases (Costerton et al. 1999). Biofilms are a primary concern in the public drinking water and energy industries. Biofilm formation reduces heat transfer efficiency of heat exchangers, increases fuel consumption of ships, enhances corrosion and blockage of water distribution pipes, fouls reverse-osmosis membranes, and contaminates food processing equipment (Jain 2009). However, biofilms offer several benefits in various other processes including biocontrol of plant pathogens (Emmert and Handelsman 1999), corrosion inhibition (Zuo 2007), wastewater treatment (Qureshi et al. 2005), bioremediation (Singh et al. 2006), productive biocatalysis (Rosche et al. 2009), and microbial fuel cells (MFCs) (Erable et al. 2009).

During biofilm formation, microbial cells undergo significant changes at the gene expression level (Gilmore et al. 2003). The changes in gene expression (genomics) lead to changes in overall metabolism of biofilm cells as compared to planktonic cells (Gjersing et al. 2007). These changes offer several unique properties to biofilms, which are either absent or poorly expressed in planktonic cells, for example, enhanced production of extracellular polymeric substances (EPS), typical three-dimensional biofilm architecture (structure), antimicrobial resistance, quorum sensing (Davies et al. 1998), gene transfer (Roberts et al. 1999), and external electron transfer (Schroder 2007). The biofilm mode of life also provides protection against osmotic stress, dehydration, and nutrient limitations to the microbial population. Biofilm allows for various physical, chemical, and molecular interactions within its resident microbial population, which enable optimal utilization of available resources (Shirtliff et al. 2002). Most biofilm-based technologies exploit and/or are determined by one or more of the above biofilm properties.

The use of biofilms as biocontrol agents and corrosion inhibitors has been described for decades. There are very few commercially available biofilm-based biocontrol agents; increased development is needed to encourage more widespread application of this technology (Morikawa 2006). Also, most biofilm-based corrosion inhibitions have been described under laboratory conditions, with few field trials (Zuo 2007). More recently, the use of biofilms in energy conversion (Erable et al. 2009) and biocatalysis processes (Rosche et al. 2009) has been described. Microbial fuel cells (MFCs) are devices that transform chemical energy into electrical energy via electrochemical reactions involving biofilms located either in both anode and cathode compartments, or in just one compartment. Ideally, biofilms in MFCs completely oxidize organic matter such as carbohydrates, cellulose, and fatty acids.

MFCs have shown promise in the field of bioremediation (Li et al., 2008), energy recovery from industrial and municipal wastewater (Raghavulu et al. 2010), and biosensor development (Di Lorenzo et al. 2009). While substantial progress has been documented under laboratory conditions, only a few pilot-scale applications of this technology are known (Keller and Rabaey 2008). Biofilm technologies have shown enormous potential in productive biocatalysis or biotransformation (Qureshi et al. 2005) and bioremediation of xenobiotics, hydrocarbons, and heavy metal pollutants (Singh et al. 2006). In biofilm reactors, the biofilms are formed on support media either by using the natural microbial populations (as in wastewater treatment) or by inoculating a desired culture (as in productive catalysis or bioremediation) in the reactor vessel. The biofilm formed on the support medium acts as self-regenerating catalyst to perform the desired reaction(s). The main advantages of biofilm treatment with respect to conventional technologies using free-floating microorganisms (e.g., activated sludge) are the higher concentration of active biomass and the resistance to short-term toxic shock, which are common issues in water treatment.

Biofilm research has received impetus by recent interest in sustainable and low-cost technologies. Microorganisms in biofilms are essentially ideal catalysts, since they can self-repair and sustain harsh environmental conditions (Rosche et al. 2009). While enzyme-based technologies can achieve faster conversion rates than those observed using viable microorganisms, enzymes are subject to rapid loss of activity under real-world process conditions. These complications increase cost and reduce environmental sustainability. Current research focuses on bridging the gap between laboratory research and industrial applications. It can be expected that the more stringent energetic and economic requirements in years to come will attract the interest of major industries toward biofilm-related technologies.

3.2 Biofilm: A Definition

A biofilm is defined as "a microbially-derived sessile community characterized by cells that attach to a substratum or interface or to each other, with the help of gelatinous extracellular polymeric substances." The special gelatinous extracellular adhesive is known as "biofilm matrix" (Costerton et al. 1999). The biofilm matrix provides protection against environmental changes, biocides, and antibiotics. Furthermore, it forms a nutrient-rich "microniche" for bacterial cells inside the biofilm by capturing and concentrating essential nutrients such as carbon, nitrogen, and phosphorus.

3.3 Mechanism of Biofilm Formation

As soon as surfaces are exposed to an aqueous environment, adsorption of dissolved organic matter onto surfaces takes place; such matter is termed conditioning film. Conditioning film is mainly composed of glycoproteins, humic material, proteins, lipids, nucleic acids, polysaccharides, and aromatic amino acids (Jain and Bhosle 2009).

The formation of conditioning film changes the overall physicochemical properties of the surface including surface charge, wettability, hydrophobicity, and surface roughness, thereby effecting bacterial adhesion. Initially, bacterial cells are weakly held to the conditioned surface by physical attractive forces such as Van der Waals forces and electrostatic forces. However, after a few hours of contact with the surface, bacterial cells begin to form more secure bonds. Subsequently, bacterial cells become firmly attached to the surface as a result of synthesis of extracellular adhesive materials. Once the cells have become firmly attached to the surface, they start multiplying. This growth is followed by overproduction of the EPS that hold the dividing cells together and support the formation of a typical mature biofilm. The mature biofilm is characterized by the presence of voids or water channels, cells embedded in the self-secreted polymeric matrix, and a characteristic three-dimensional structure (Lewandowski 2000). These voids or water channels allow water and nutrients to migrate to deeper portions of mature biofilm. In order to colonize new surfaces and to avoid population density-mediated starvation, bacterial cells are transported from biofilm phase to liquid phase by a process known as biofilm detachment. Continuous biofilm detachment maintains balance in biofilm growth, enabling the biofilm thickness to reach a pseudo steady state.

3.4 Biofilm Properties: Influence on Biofilm-Based Technologies

A specific biofilm (mixed species or pure culture biofilm) is selected for the development of biofilm-based technologies on the basis of its desired properties; some are discussed below.

3.4.1 Extracellular Polymeric Substances: Role in Biofilm Reactor Performance

Bacterial cells irreversibly adhere to surfaces or each other through the production of EPS. EPS consist of water, polysaccharides, proteins, humic substances, nucleic acids, and lipids (Bhosle et al. 1996). EPS play a significant role in biofilm formation and structure (Jain and Bhosle 2008), and biofilm EPS are important in terms of industrial biofilm application.

Accumulation of unproductive EPS within bioreactors reduces the space available for active cells and may hamper the overall volumetric productivity of catalytic biofilm processes. Recently, Setyawatia et al. (2009) have reported that high EPS content in *Acetobacter xylinum* biofilm caused mass transfer limitation and led to a sixfold decline in the biotransformation activity of the biofilm. Excessive sloughing of EPS poses difficulties for downstream processing and product purification. Therefore, during industrial biofilm application, EPS production and accumulation must be carefully monitored and controlled.

3.4.2 Biofilm Architecture: Role in Biofilm Reactor Performance

Biofilm architecture is heterogeneous both in space and time, constantly changing due to external and internal processes. Biofilm architectural characteristics such as thickness, density, and surface shape are crucial for the stability and performance of a biofilm reactor, since they affect biomass holdup and mass transfer. For example, thin biofilms (<100–150 μm) enable oxygen to diffuse into the deepest layers, while thick biofilms create an external aerobic zone and an inner anaerobic zone. Therefore, control of biofilm thickness is of paramount importance to the operation of biofilm reactors. Biofilm surface shape also plays a crucial role, since fluffy biofilms and outgrowth lead to biofilm instability [e.g., in fluidized bed reactors (FBRs)] (Tijhuis et al. 1995).

3.4.3 Quorum Sensing: Role in Bioreactor Cleanup

Bacteria produce diffusible extracellular signaling molecules, e.g., acylated homoserine lactones (AHLs; Gram-negative bacteria) and oligopeptides (Gram-positive bacteria), to monitor their population density and to coordinate expression of specific sets of genes in response to cell density. This type of cell-density-dependent gene regulation is termed "quorum sensing." Davies et al. (1998) published the first study showing the role of quorum sensing in the formation of biofilms and launched a period of research on cell-to-cell signaling in biofilms. Quorum sensing affects every aspect of biofilm development including its dispersal, which allows cells to be released from biofilms and to colonize new niches (Hall-Stoodley et al. 2004). Little is currently known about quorum sensing and biofilm dispersal in industrial biofilm processes. Once these mechanisms are better understood, it might be possible to control biofilm development in industrial applications with the aim of not only enhancing productivity but also facilitating bioreactor cleanup.

3.4.4 Antimicrobial Resistance: Role in Bioreactor Cleanup

Biofilms are well known for their enhanced tolerance to antimicrobials, toxic substances, and other adverse conditions compared to suspended cells. A number of resistance mechanisms contribute to this characteristic including lower diffusion within biofilms, limited cell growth, the formation of specialized survivor cells termed persisters, and active toxin removal (Anderson and O'Toole 2008). Some of these biofilm resistance mechanisms could be unfavorable for productive catalysis. For example, resistance conferred by limited growth could hinder the production of growth-linked metabolites in biofilms. Also, limited diffusion and removal of toxic substrates by efflux or degradation would negatively affect productivity.

3.4.5 Gene Transfer Within Biofilms: Role in Bioremediation

Gene transfer occurs frequently and effectively in many bacterial biofilms, both in natural environments and in artificial settings. Gene transfer affects a population's potential to meet and exploit new environmental conditions. Conjugation and transformation are the two ways by which gene transfer occurs in biofilms (Maiques et al. 2007). Moreover, transfer of catabolic genes in natural as well as in vitro biofilms has shown great potential in bioremediation processes (Dejonghe et al. 2000). Several gene carriers termed mobile genetic elements (MGE) (i.e., plasmids and transposons) are used for gene transfer in biofilms. The horizontal exchange of catabolic genes among bacteria in their metabolic pathways could help in the construction of novel catabolic pathways and strategies for bioremediation. The transfer of catabolic genes can be used to optimize bioreactor efficiency in cases where degradation is limited by low biomass and shortage of degradative genes.

3.4.6 External Electron Transfer in Biofilms: Role in MFC Function

A small group of biofilms, mainly formed by dissimilatory metal-reducing bacteria, can exchange electrons directly with electrodes and are therefore termed electrochemically active biofilms (EABs) (Reimers et al. 2001). EABs transfer electrons extracellularly via (1) microbially produced redox mediators, (2) membrane proteins, or (3) conductive appendages termed nanowires. Microbially produced redox mediators can undergo repetitive oxidation/reduction, thereby facilitating the transfer of electrons between biofilm and electrode. Examples of microbially produced redox mediators are phenazines (i.e., pyocyanine), quinone-related molecules, and flavins (Marsili and Zhang 2010). EAB-forming bacteria capable of direct extracellular electron transfer can switch their metabolism from a soluble electron donor (e.g., hydrogen, glucose, acetate) or acceptor (e.g., oxygen, nitrate, fumarate) to a solid electron donor or acceptor at the surface of a conductive electrode (Bond and Lovley 2005). Recently, the role of bacterial cell surface appendages (i.e., pili, often termed nanowires) has been reported in electron transfer between biofilms and electrodes (Reguera et al. 2005).

3.5 Application of Biofilms

3.5.1 Biofilms as Biocontrol Agents

Biocontrol against plant diseases using microorganisms is a powerful alternative to the use of synthetic chemicals (Morris and Monier 2003). Recent studies have suggested that the biofilm mode is important for bacteria to act as a biocontrol agent

(Bais et al. 2004). In this mechanism, the biofilm of a particular strain on plant surfaces will suppress a disease without causing long-term effects on the rest of the indigenous microbial population. Currently, few biocontrol agents are commercially available; reasons for the slow development of this technology are (1) the lack of knowledge of the biological control system and (2) the difficulty in obtaining a successful formulation (Fravel 2005). Most biocontrol agents have been successful in laboratory trials; however, stability, effectiveness of the formulation, and scale-up production remain cumbersome. For example, viable formulations of fluorescent *Pseudomonads* experience a major obstacle for their large-scale field application. Sporulating Gram-positive microorganisms such as *Bacillus* and *Streptomyces* may help to solve the product formulation problem. These bacteria form heat- and desiccation-resistant spores that can be converted readily into stable products such as dry powder (Morikawa 2006).

3.5.1.1 Gram-Positive Bacterial Biofilms as Biocontrol Agents

Bacillus subtilis and *Bacillus cereus* are often found in the plant rhizosphere, i.e., the biologically and chemically active area of soil surrounding the plant root. In the rhizosphere, both *B. subtilis* and *B. cereus* promote plant growth and act as biocontrol agents (Bais et al. 2004). The commercial biocontrol agent Serenade, which contains a *B. subtilis* strain, is highly effective against a variety of pathogenic bacteria including *Erwina*, *Pseudomonas*, and *Xanthomonas* strains (Fravel 2005), whereas *B. cereus* has been used commercially for reliable biocontrol of various phytopathogens, especially oomycete pathogenic fungi. Upon root colonization, *B. subtilis* and *B. cereus* form stable and extensive biofilms. *B. subtilis* biofilm produces a variety of antibacterial agents including a broad range of lipopeptides such as surfactin, which are potent biosurfactants and important for maintaining the aerial structure of biofilms (Ongena and Jacques 2008). *B. cereus* biofilm produces two antibiotics, zwittermicin A and kanosamine, which contribute to biocontrol of alfalfa damping. The commercial success of these organisms as biocontrol agents is due to the ease of formulation and storage of the products.

3.5.2 Biofilms as Corrosion Inhibitors

Corrosion inhibition is the suppression of the corrosion reaction by inhibitors, either abiotic or biotic. The nature of corrosion inhibition is electrochemical, i.e., the same general mechanism as for corrosion (Videla and Herrera 2009). Mechanisms of corrosion inhibition are complex and often involve more than the production of a simple barrier on the metal. Recently, Zuo (2007) has reviewed recent progress in corrosion inhibition by beneficial bacterial biofilms and proposed four different mechanisms. These are discussed in detail below.

3.5.2.1 Corrosion Inhibition by Biofilm Through Oxygen Removal

Corrosion inhibition by biofilm-forming bacteria is a general phenomenon, and the nature of the biofilms (i.e., good biofilm former or poor biofilm former) determines the degree of corrosion inhibition. Additionally, an increase in depth of biofilm is known to correspond to a greater reduction in corrosion (Jayaraman et al. 1999a). Moreover, bacterial metabolic activities contribute to oxygen removal by biofilms (Dubiel et al. 2002).

3.5.2.2 Corrosion Inhibition by Biofilms Secreting Antimicrobials

Corrosion control approaches using biofilms secreting antimicrobials are known as corrosion control using regenerative biofilms (CCURB) (Zuo et al. 2004). This strategy enables the production of antimicrobial compounds within the biofilm (naturally or genetically constructed). Moreover, EPS help to maintain relatively higher local antimicrobial concentrations by preventing them from diffusing into bulk fluids. Jayaraman et al. (1999b) genetically constructed *B. subtilis* and *B. brevis* biofilms secreting antimicrobials and found that these biofilms inhibited the growth of corrosion-causing sulfate-reducing bacteria (SRB) as well as reduced corrosion rates significantly. Zuo et al. (2004) conducted a field trail evaluating the impact of protective gramicidin-S-producing *B. brevis* biofilm against multiple corrosive bacteria and reported a two- to tenfold decrease in the corrosion rate of mild steel.

3.5.2.3 Corrosion Inhibition with Biofilms Secreting Corrosion Inhibitors

Biofilms are ideal for delivering corrosion inhibitors, as they are generated within the biofilm and are adjacent to metal surfaces. Mansfeld et al. (2002) investigated the efficiency of polyaspartate or γ-polyglutamate (naturally or genetically constructed within the microorganism) as corrosion inhibitors. Both polyaspartate and γ-polyglutamate chelate metals, thereby protecting the metal surface from uniform corrosion by formation of an aluminum/anionic peptide complex.

3.5.2.4 Corrosion Inhibition Through Protective Layers (Biofilm Matrix)

Protective layers may be a passive oxide product formed during corrosion, which becomes entrapped in the biofilm matrix. Chongdar et al. (2005) reported that aerobic *Pseudomonas cichorii* was able to inhibit corrosion of mild steel due to formation of an iron oxide/iron phosphate layer within the biofilm matrix. Juzeliunas et al. (2006) isolated *Bacillus mycoides*, a Gram-positive, nonmotile soil bacterium that increases the charge transfer resistance of the aluminum, thereby inhibiting its corrosion.

3.6 Biofilm-Based Technologies

3.6.1 Biofilm Reactors

Biofilms can be used in various types of reactors such as continuous stirred tank reactors (CSTRs), packed bed reactors (PBRs), trickling bed reactors (TBRs), FBRs, airlift reactors (ALRs), upflow anaerobic sludge blanket (UASB) reactors, expanded granular sludge beds (EGSB), internal circulation (IC) reactors, and membrane aerated biofilm reactors (MABR). High reaction rates are the main advantage of biofilm reactors vs. conventional suspended biomass reactors (Table 3.1).

A CSTR consists of a vertically baffled tank fitted with a mechanical stirrer. It is continuously fed with influent, and treated effluent is removed from the reactor at the same rate (Fig. 3.1a). The PBR (Fig. 3.1b) uses a variety of packings (porous and nonporous) for biofilm growth, including polymers, ceramic, glass, and natural material (wood or bark). The TBR (Fig. 3.1c), in contrast to PBR, employs counterflow of gas and liquid through packing material or beds. TBRs are used for large-scale production of vinegar, as biofilters for gas cleanup and deodorization, for water purification, and for ore leachate treatment. In FBRs, microbial cells grow around adsorbent particles and form active biofilms. FBRs can maintain high

Table 3.1 Comparative evaluation of biofilm-based reactors with other types of biological reactors

	Reactor type			
Attributes	Biofilm based	Entrapment based	Immobilized reactors	Membrane reactors
Advantages	Comparatively highest reactor productivity	High productivity	High productivity	High productivity
	High cell concentration is achieved	High cell concentration may be achieved	High cell concentration may be achieved	High cell concentration may be achieved
	Reactors can run longer			
	Economical operation			
Disadvantages	Effluent centrifugation required	Effluent centrifugation required	Effluent centrifugation required	Membrane fouling
		Disintegration of matrix material with time	Restricted cell growth inside matrix	Not economical for the large volumes
		Cell leaching from matrix	Chemical may affect cell growth	

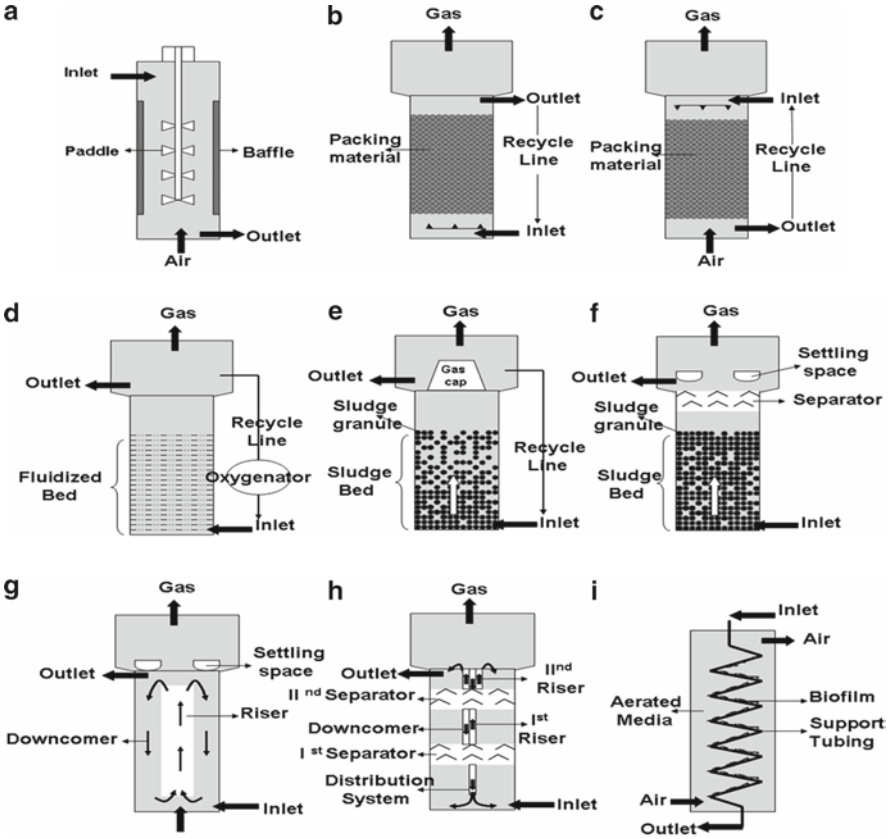

Fig. 3.1 Schematic representation of various types of biofilm reactors. (**a**) continuous stirred tank reactor (CSTR); (**b**) packed bed reactor (PBR); (**c**) trickling bed reactor (TBR); (**d**) fluidized bed reactor (FBR); (**e**) upflow anaerobic sludge blanket reactor (UASB); (**f**) expanded granular sludge bed reactor (EGSB); (**g**) air lift reactor (ALR); (**h**) internal circulation (IC); and (**i**) membrane aerated biofilm reactor (MABR)

volumetric concentrations of biomass and a high substrate conversion rate. In FBRs (Fig. 3.1d), the flow of liquid is directed through the particles above the "minimum fluidization velocity," leading to the lifting of particles from their fixed bed. FBR can be operated for much longer times (more than 4 months) than PBRs or CSTRs (with fibrous bed) without experiencing blockage. UASB reactors are based on the use of granular biofilm particles and are used for the anaerobic treatment of wastewater and industrial effluents. At the top of the reactor, provisions are made for gases to escape and sludge particles to settle at the bottom of the reactor (Fig. 3.1e). In order to improve process efficiency of UASB reactors, an adequate influent distribution is required. The use of effluent recirculation in combination with a taller reactor (a larger height-to-diameter ratio) results in the development of EGSB reactors. EGSB reactors are also used for the anaerobic treatment of wastewater/

industrial effluents (Fig. 3.1f). ALRs use circular mixing achieved by gas injection (Fig. 3.1g). This results in a lower degree of shear and more vigorous recirculation for the same rate of airflow. ALRs contain two concentric tubes, a riser (an inner tube) and a downcomer (an outer tube). In an IC reactor (Fig. 3.1h), influent is pumped at the bottom and is mixed with the granular anaerobic biomass. In the lower reactor compartment, most of the organic components are converted to methane and carbon dioxide. The self-regulating IC reactor offers considerable advantages in system operation, leading toward reduced operational costs and increased productivity and reliability. The MABR uses immobilized microbial biomass on membranes through which oxygen is supplied (Fig. 3.1i). MABRs minimize losses of volatile organic compounds and allow easy control of oxygen penetration into biofilms through the membranes. The dissolved oxygen gradient across the membrane and the biofilm offers an ideal environment for aerobic microbial strains and prevents foaming in the reactor due to generation of surfactants. Moreover, enhanced oxygen penetration makes MABRs an attractive option for pollutant biodegradation in high-strength wastewaters.

3.6.1.1 Biofilm Reactors in Wastewater and Waste Gas Treatment

The application of various types of biofilm reactors in wastewater treatment has been extensively reviewed elsewhere (Qureshi et al. 2005). Table 3.2 describes some of the important applications of biofilm reactors in wastewater treatment.

Biological treatment of waste gas is an attractive and environmentally friendly alternative to physicochemical methods. The biofilter, trickling biofilter, and bioscrubber are the three major bioreactor designs frequently employed for treatment of waste gas. A biofilter consists of a filter bed composed of a support material (sawdust, compost, dry wastewater sludge, etc.) for the active microorganisms and as a nutrient source. Waste air is blown through the biofilms around particles of biofilter medium. On the other hand, in trickling biofilters, waste gas is continuously fed with a liquid medium to the biofilm grown on the packing material. The bioscrubber is mainly used for treatment of waste gases containing high concentrations of water-soluble pollutants.

3.6.1.2 Biofilm Reactors in Bioremediation Process

Bioremediation is an emerging, efficient, and economical in situ technology that employs microorganisms for the cleanup of environmental pollutants (Woodley 2006). Biofilms are well suited for treatment of recalcitrant or slow-degrading compounds because of their high microbial biomass and ability to immobilize compounds by biosorption, bioaccumulation, and biomineralization (Table 3.3). Physiological properties of microorganisms such as biosurfactant production and chemotaxis enhance bioavailability and degradation of hydrophobic compounds (Paul et al. 2005).

Table 3.2 Application of biofilm reactors in wastewater treatment

Type of medium	Basic features	Applications in wastewater treatment	References
Fixed medium	Biofilm media are static in the reactors		
Trickle bed	Biofilms are grown on static media (stone, gravel)	Used for large-scale anaerobic wastewater treatment	Rodgers and Zhan (2003)
Membrane aerated filters	Biomass is immobilized on membranes.	Losses of volatile organic compounds are minimized Ideal for treating high-strength wastewaters	Woolard and Irvine (1994)
Moving medium	Biofilm media are kept continually moving by means of mechanical, hydraulic, or air forces		
Rotating biological contactors	Treats wastewater streams using a thin biofilm of aerobic microorganisms on rotating cylinders or biodiscs	Used in biological treatment of wastewater Reduces chemical oxygen demand (COD), biological oxygen demand (BOD) Used in nitrification and denitrification	Kargi and Dinçer (1999)
Fluidized bed reactors	Particles move up and down within the expanded bed in the well-defined zone of the reactor	Ten times more efficient than activated sludge process Occupies 10% of the space required by stirred tank reactors	Rabah and Dahab (2004)
Upflow sludge blanket	Formation of a dense sludge bed in the bottom of the reactor due to microbial growth and incoming sludge	Anaerobic removal of various chemicals from the wastewater (e.g., pentachlorophenol, nitrogen, phenol, and cresols); dechlorination, starch degradation	Schmidt et al. (2004)
Expanded Granular sludge	More efficient than UFSB because of adequate influent distribution		Tsuno et al. (1996)

Table 3.3 Examples of commercially available biofilm reactors and their industrial-scale applications in wastewater treatment

Type of reactor	Commercial name	Treatment process	Commercial application	References
Biofilm-fluidized bed (BFB)	ANAFLUX (Degremont France)	BIOLITE R-280 as fluid bed media to treat a variety of brewery, food-processing, and paper-industry wastewater	Starch factory, Habourdin, France (1993)	Holst et al. (1997)
	OXYTRON, ANYTRON (Dorr-Oliver, USA)	Carbonaceous oxidation, nitrification, denitrification, and anaerobic reduction of municipal and industrial automotive industry, coke-making operations, wastewater treatment using sand and activated carbon as fluid bed media	Coke plant, ON, Canada (1996)	
Upflow anaerobic sludge blanket reactors (UASBs)	BIOPAQ (Paques, The Netherlands)	Anaerobic treatment of high-strength wastewaters	Distillery, Wellington, South Africa (1996)	Wolmarans and Driessen (1996)
Expanded granular sludge blanket (EGSB)	BIOBED (Biothane, USA)	Anaerobic treatment of high-strength wastewaters (wastewater from the Netherlands food, brewery, yeast pharmaceuticals, paper, and chemical industry)	Rotterdam, The Netherlands (1993)	Zoutberg and Frankin (1996)
Internal circulation (IC)	Paques, The Netherlands	Anaerobic treatment of high-strength wastewaters	Rosendaal, The Netherlands (1995)	Habets et al. (1997a, b)
Airlift reactors (ALRs)	BAS CIRCOX, Paques, The Netherlands ASTRASAND, Paques, The Netherlands	Applied to pharmaceutical and brewery wastewaters, which are anaerobically pretreated, and to municipal wastewater Biological (de)nitrification and polishing of municipal and industrial waste water	Brewery, Enschede, The Netherlands (1994)	Gorur et al. (1995)
Anaerobic flotation reactor (AFR)	BIOPAQ (Paques, The Netherlands)	Especially developed for the anaerobic treatment of waste streams that contain FOG (fat, oil, grease) and/or biodegradable solids like proteins and starch	http://www.paques.nl/	

Table 3.4 Biodegradation of chlorophenols, azo dyes, and herbicides using different biofilm reactors

Methods of remediation	Experimental conditions	Heavy metals remediation	References
Biosorption	Anaerobic–anoxic biofilm process	Zn, Cd, Ni	Chang et al. (2006)
	Biofilm formed on moving bed sand filter	Cu, Zn, Ni, Co	Diels et al. (2003)
	Biofilm formed on kaolinite	Fe, Cd, Ni, Cr	Quintelas et al. (2009)
Immobilization	Rotary biofilm reactor for algae immobilization	Co	Travieso et al. (2002)
Adsorption	Biofilm development over granular activated carbon	Cd, Cu, Zn, Ni	Scott and Karanjkar (1998); Scott et al. (1995)
Bioprecipitation	Bacteria immobilized composite membrane reactor	Cd, Zn, Cu, Pb, Co, Ni, Pd, Ge	Diels et al. (1995)
	Biogenic H_2S from sulfate-reducing biofilm	Zn, Cu, Pb	Alvarez et al. (2007)

Bioremediation of Hydrocarbons

Chlorinated aromatic compounds (recalcitrants) are widespread contaminants of soil and groundwater, and many are carcinogenic even at extremely low concentrations. Table 3.4 lists the bioreactor types used in the bioremediation of hydrocarbons. Kargi and Eker (2005) reported complete degradation of 2,4-dichlorophenol (DCP) using a rotating perforated tube biofilm reactor with a mixed microbial biomass supplemented with DCP-degrading *Pseudomonas putida*. Johnsen et al. (2005) reported that polycyclic aromatic hydrocarbons (PAHs) accumulate in bacterial biofilms by adsorption to microbial exopolymers. Subsequently, during periods of starvation, the biofilm community metabolizes the accumulated PAHs. Perumbakkam et al. (2006) introduced atrazine-degrading genes into biofilms of *Acinetobacter* sp. BD413, thereby developing a biofilm-mediated process to degrade atrazine. Improving strains by engineering metabolic pathways and enzymes involved in degradation or by increasing the number of copies of degradative genes could further enhance biofilm-mediated bioremediation; therefore, a combination of genetic engineering of microorganisms and optimization of physicochemical parameters and substrate concentrations in bioreactors is of paramount importance for developing bioremediation strategies.

Bioremediation of Heavy Metals

Another promising application of biofilms is in heavy-metal and radionuclide remediation. Table 3.5 lists the biofilm reactor types used in bioremediation of heavy metals. Biofilm-assisted heavy-metal bioremediation can be achieved by

Table 3.5 Bioremediation of heavy metals using biofilm bioreactors

Type of bioreactor	Pollutant chlorophenols	Organism or culture	References
Silicone tube membrane bioreactor	2-Chlorophenol	Anaerobic sludge from a swine waste treatment plant	Chang et al. (2003, 2004)
Granular activated-carbon biofilm reactor	4-Chlorophenol	Bacterial consortium from rhizosphere of *Phragmites australis*	Caldeira et al. (1999); Carvalho et al. (2001)
Rotating perforated tube biofilm reactor	2,4-Dichlorophenol	*Pseudomonas putida*	Kargi and Eker (2005)
Fluidized bed biofilm reactor	2,4,6-Trichlorophenol, 2,3,4,6-tetrachlorophenol, pentachlorophenol	*Pseudomonas* sp. *Rhodococcus* sp.	Puhakka et al. (1995)
Biofilm grown directly on liquid medium	Pyrene, phenanthrene	*Polaromonas* sp., *Sphingomonas* sp., *Alcaligene* sp., *Caulobacter* and *Variovorax* sp.	Eriksson et al. (2002)
Biofilm grown in NAPLs	0-Cresol, naphthalene, phenol, 1,2,3-trimethly benzene	*Pseudomonas fluorescens*	Vayenas et al. (2002)
Rotating biological contactors	*n*-Alkanes	*Prototheca zopfii*	Yamaguchi et al. (1999)
Continuous flow fixed biofilm reactor	Carbon tetrachloride	*Providencia stuartii, Pseudomonas cepacia*	Jin and Englande (1998)
Hollow-fiber membrane biofilter reactor, continuously fed biodrum reactor	Toluene	Secondary sludge from waste water treatment plant, various aerobic and anaerobic bacteria	Parvatiyara et al. (1996); Arcangeli and Arvin (1995)
	Azo dyes		
Laboratory-scale rotating drum biofilm reactor	Acid orange 10,14	*Methylosinus trichosporium*	Zhang et al. (1995)
Laboratory-scale activated sludge unit	Everzol Turquoise	*Coriolus versicolor*	Kapdan and Kargi (2002)
	Herbicides		
Granular activated-carbon biofilm reactor	2-(2-Methyl)-4-chlorophenoxyl propionic acid (MCPP)	Mixed culture of herbicide degrading bacteria	Oh and Tuovinen (1994)

immobilization, biosorption, and concentration, thereby minimizing anticipated hazards (Quintelas et al. 2009). There are several reports suggesting the role of biofilm EPS in metal precipitation, for example, accumulation of polycrystalline $NaUO_2PO_4$ by *Citrobacter* sp. N14 (Macaskie et al. 2000), and formation of sphalerite (ZnS) by members of Desulfobacteraceae in a natural biofilm (Labrenz et al. 2000). The construction of improved strains with specific metal-binding properties through expression of metal-chelating proteins and peptides can improve metal precipitation processes.

3.6.1.3 Biofilm Reactors in Productive Biocatalysis

Biocatalysis is an effective and environmentally friendly tool for industrial production of chemicals (Woodley 2006). Biocatalysts including bacteria, fungi, and their enzymes can be produced from renewable resources, are biodegradable, and catalyze reactions with high specificity under normal conditions. Cell retention during continuous bioreactor operation and long-term cell viability are the main challenges in effective productive biocatalysis, and microbial biofilms offer solutions to these problems. Biofilms produce their own immobilization matrix and are naturally adapted to be viable within the matrix. This offers the possibility of continuous reactor use and significant cost reduction (per kg of product) compared to conventional systems (Gross et al. 2007). Owing to their robustness and ease of operation, various biofilm processes have already been implemented commercially with great success over the last few decades. An example is the commercial production of vinegar using acetic acid bacteria (El-Mansi and Ward 2007). Qureshi et al. (2005) reviewed the feasibility of biofilm reactors in the conversion of agricultural materials such as starch, sugars, and glycerol into alcohols (e.g., ethanol, butanol, 2,3-butanediol, dihydroxyacetone) or organic acids (e.g., acetic, lactic, fumaric, succinic, and citric acid). Recently, Wang and Chen (2009) have reviewed the various opportunities for biofilm-based biofuel production. Further research in the field has been directed toward investigating biofilm potential for the production of other useful chemicals.

The examples shown in Table 3.6 illustrate the range of reactor configurations, surface materials, and organisms that has been considered for biocatalysis, mostly on a laboratory scale. Most recently, biofilm processes involving toxic substrates and toxic products have been the focus of attention. In this context, Li et al. (2006) reported that biofilms of *Zymomonas mobilis* were more tolerant to the toxic substrate benzaldehyde than free-floating cells of the same strain and were able to produce benzyl alcohol in a continuous biofilm reactor. Gross et al. (2007) developed a tubular biofilm reactor for bioconversion of styrene, to (S)-styrene oxide, which is a biologically challenging compound because it is volatile, poorly water-soluble and toxic to cells. A number of other studies have also reported successful application of biofilms in productive catalytic processes without biocatalyst degeneration or contamination over several months, demonstrating the potential of biofilm applications for industrial catalysis.

Table 3.6 Application of biofilm-based reactors in productive biocatalysis

Type of reactor	Surface material	Organism	Substrate	Product	References
Trickling bed (TBR)	Beechwood shavings	Acetic acid bacteria	Ethanol	Acetic acid (vinegar)	Crueger and Crueger (1990)
	Polyurethane foam	*Caldicellulosiruptor saccharolyticus*	Sucrose	Hydrogen	van Groenestijn et al. (2009)
Packed bed	Polypropylene	*Zymomonas mobilis*	Glucose	Ethanol	Kunduru and Pometto (1996)
	Glass beads	*Zymomonas mobilis*	Benzaldehyde	Benzyl alcohol	Hekmat et al. (2007)
	Cotton fabric	*Lactococcus lactis*	Lactose	Nisin	Liu et al. (2005)
	Anion exchange resin	*Alcaligenes eutrophus*	Glucose	Poly(3-hydroxybutyrate)	Zhang et al. (2004)
	Silicone-coated Ralu rings	*Gluconobacter oxydans*	Glycerol	Dihydroxyacetone	Hekmat et al. (2003)
Fluidized bed	Macroporous glass beads	*Zymomonas mobilis*	Hydrolyzed starch	Ethanol	Weuster-Botz et al. (1993)
Membrane reactor	Membrane	Anaerobic microorganisms	Syngas	Liquid products, e.g., ethanol and butanol	Hickey et al. (2008)
Tubular membrane reactor	Silicone tubing	*Pseudomonas* sp.	Styrene	(S)-Styrene oxide	Gross et al. (2007)
Semicontinuous stirred tank reactor	None; bacteria create cellulose granules	Recombinant *Acetobacter xylinum*	D-Alanine	Pyruvic acid	Steinberger and Holden (2004)
Continuous stirred tank reactor	Fibrous bed	*Clostridium acetobutylicum*	Corn	Butanol	Huang et al. (2007)
Stirred tank reactor	Fibrous bed	*Rhizopus oryzae*	Glucose, starch	Lactic acid	Tay and Yang (2002)

3.6.2 *Microbial Fuel Cells*

MFCs convert chemical energy stored in reduced carbon compounds directly into electrical energy via microbial catalysts (Liu et al. 2004). Although MFC design depends largely on the application, a conventional MFC is composed of two chambers, an anode and a cathode, separated by a cation exchange membrane (Fig. 3.2a). Electroactive biofilms oxidize the substrate at the anode and produce electrons and protons. The electrons are transported via an external circuit to the cathode, while the protons are transferred through the cationic membrane. At the cathode, a terminal electron acceptor (i.e., oxygen) is reduced.

The power output of a MFC is affected by environmental conditions such as presence of oxygen and by the availability of an easily degradable carbon source. MFCs are fuelled by a wide variety of substrates including glucose, acetate, sucrose, ethanol, butyrate, glutamate, and wastewater. MFC designs are rapidly changing; for example, the cationic membrane has been removed to reduce the costs (Liu and Logan 2004), and both anodic and cathodic material are being investigated to increase electroactive

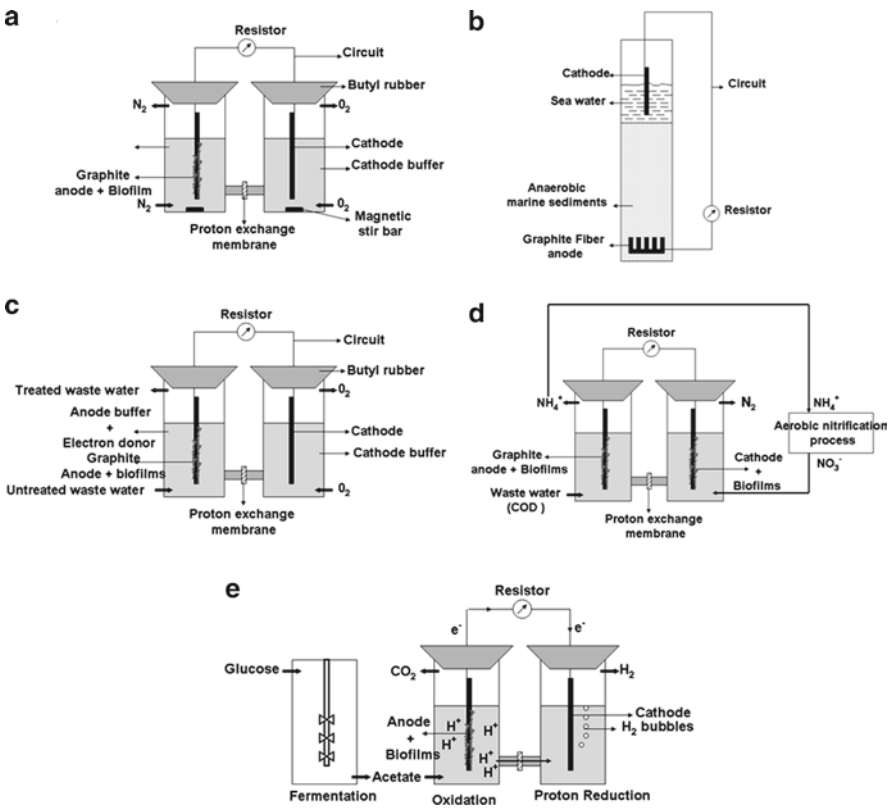

Fig. 3.2 Schematic diagram of two chamber microbial fuel cells. (**a**) basic design of MFC; (**b**) marine MFC; (**c**) wastewater MFC; (**d**) biocathode MFC; and (**e**) biohydrogen-producing MFC

biofilm concentration and its extracellular electron transfer rate. The adoption of a single-chamber MFC, wherein the reduction occurs directly in the gas phase increases coulombic efficiency of MFCs and reduces its technical complexity.

3.6.2.1 Marine MFCs

Marine MFCs typically consist of a graphite anode embedded in anaerobic marine sediments and are connected through an electrical circuit to a cathode setup in the overlying aerobic seawater as shown in Fig. 3.2b (Reimers et al. 2001). In marine MFCs, a constant supply of fuel and oxidants, by environmental processes such as settlement of dead phytoplankton and/or vegetative detritus, results in constant regeneration of microbial electrode catalysts. MFCs implemented in marine sediments show sustained current production over long periods (Reimers et al. 2006). Tender et al. (2008) demonstrated, for the first time, the use of marine MFC as a practical alternative to batteries for low power-consuming applications such as buoys and marine sensors.

3.6.2.2 Wastewater MFCs

MFCs can be operated successfully on a variety of substrates including complex materials such as domestic wastewater (Ghangrekar and Shinde 2008) (Fig. 3.2c), swine manure slurry (Min et al. 2005), landfill leachate (You et al. 2006), and meat-packing wastewater (Heilmann and Logan 2006). Individual cell voltages of 0.7 V have been reported by researchers with power densities varying between 20 and 2,000 mW/m^2 of anode surface area, depending upon configuration of MFC, substrate, anodophilic microorganisms, and operating conditions used. Integration of MFCs will enable significant economy in wastewater treatment (Logan et al. 2008).

3.6.2.3 Farm Field MFCs

Another interesting application of MFCs is in farm fields to generate power from cultivated plants. Electrical current was generated via in situ oxidation of rhizodeposits from living rice plants. The electrical power output of a sediment MFC was found to be a factor of seven higher in the presence of actively growing plants. This process offers the potential of light-driven power generation from living plants in a nondestructive way. Sustainable power production up to 330 W/h may be attributed to the oxidation of plant-derived compounds (De Schamphelaire et al. 2008). This technology can provide small amounts of power in remote, off-grid locations.

3.6.2.4 Photosynthetic MFCs

In this relatively recent technology, the combination of microalgae and EABs has enabled a production of 110 mW/m^2 (Strik et al. 2008). Microalgae are particularly interesting for energy conversion processes because they require relatively little

organic carbon and nutrients for their maintenance. Recently, Rosenbaum et al. (2010) have reviewed the five approaches that integrate photosynthesis with MFCs.

3.6.2.5 Applications of MFCs

Researchers are showing increasing interest in the application of MFCs into various fields including wastewater treatment, bioremediation, metal oxidation, and biosensor and biohydrogen production. MFCs are used for the removal of oxidizable matter from industrial as well as domestic wastewaters. The published results (Table 3.7) demonstrate the utility of MFCs as a wastewater treatment system.

Table 3.7 Application of electroactive biofilms in a variety of systems including wastewater treatment, biocathodes, bioremediation, and biohydrogen production

Application of electroactive biofilms	Results	References
Wastewater treatment	72% COD removal from wastewater	Min and Logan (2004)
	80% COD removal from domestic wastewater	Liu et al. (2004)
	91% COD removal form swine wastewater	Min et al. (2005)
	93–95% COD removal from starch, peptone, and fish extract wastewater	Shimoyama et al. (2008)
	53% Reduction in biochemical oxygen demand (BOD) of wastewater	Greenman et al. (2009)
Biocathode in current production	Fourfold increase in the current output	Freguia et al. (2008)
	SS cathode resulted in higher current than graphite	Dumas et al. (2008)
	Biocathode can oxidize ammonia and helps in denitrification	Holmes et al. (2004)
Biocathode in nitrogen removal	Biocathode as the sole electron donor for nitrate reduction to nitrite	Gregory et al. (2004)
	Reduction of nitrate at the biocathode	Clauwaert et al. (2007)
	Removal of 0.41-kg equivalent nitrogen/m^3 using biocathode per day	Virdis et al. (2008)
Metal oxidation	Manganese oxidation using *Leptothrix discophora*	Rhoads et al. (2005)
	Chromium removal with a power density of 1,600 W m72	Li et al. (2008)
Biosensor development	Biosensor for in situ monitoring of nitrate/nitrite concentrations	Larsen et al. (2000)
	Linear correlation between current and nitrate/nitrite concentration	Nielsen et al. (2004)
	Linear response in current with increase in glucose concentration from 0 to 25 g/l	Kumlanghan et al. (2007)
	Current produced by EAB can be correlated to the available substrate concentration and the EAB respiration rate	Tront et al. (2008)

(continued)

Table 3.7 (continued)

Application of electroactive biofilms	Results	References
Biohydrogen production	Biohydrogen production using EABs	Rozendal et al. (2006)
	Biohydrogen production using complex organic molecules such as glucose, cellulose, and different volatile acids as the substrate	Cheng and Logan (2007)
	Single chamber, membrane-free MECs for biohydrogen production	Hu et al. (2008)
	Linear correlation between voltage and H_2 generation	Call and Logan (2008)

While scale-up and long-term feasibilities of MFCs remain a complex issue (Dewan et al. 2008), it is reasonable to expect that MFCs will gradually replace complex and energetically unsustainable technologies currently in use for wastewater treatment. Recently, it has been reported that the biocathode in MFCs can harvest considerable energy from pollutants in wastewater. Biocathodes can act as efficient catalysts for oxygen reduction and are a feasible alternative to abiotic systems in wastewater-fed MFCs. Holmes et al. (2004) found that a biocathode system of benthic MFCs participated in biological reactions such as ammonia oxidation and denitrification. Virdis et al. (2008) described an MFC configuration for simultaneous nitrification and dentrification in separate anodic and cathodic chambers. The anode effluent was aerated externally for nitrification to occur, and the nitrified effluents were routed to the cathode for denitrification (Fig. 3.2d).

Rhoads et al. (2005) employed a manganese-oxidizing bacterium (MOB), *Leptothrix discophora*, which oxidized Mn_2 to MnO_2 by releasing two electrons to oxygen. Li et al. (2008), using graphite paper as a cathode, demonstrated successful chromium removal with a maximum power density of 1,600 W m72 at a Columbic efficiency of 12%. The integration of MFC in the water electrolysis process enabled the system to reduce energetic costs for hydrogen generation. This technology is termed microbial electrolysis cells (MECs) (Logan et al. 2008). In a typical MEC, a small voltage is applied between anode and cathode, and the current generation is forced, thereby resulting in hydrogen production at the cathodic side due to proton reduction (Fig. 3.2e). Due to a thermodynamic barrier, many organic compounds are unsuitable for use as a substrate in fermentative hydrogen production (Logan et al. 2008). However, they can be used in MECs because in this case, a small external voltage is applied to overcome the thermodynamic barrier (Holladay et al. 2009).

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Chapter 4
Future Application of Probiotics: A Boon from Dairy Biology

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Abstract Microbes have been used for millenia in food and alcoholic fermentations; in recent years, microbes have undergone scientific scrutiny of their ability for preventive and therapeutic effects in humans. This work has led to the establishment of a new term, "probiotics." Lactic acid bacteria (LAB) are normal microflora of the intestine of most animals. They play an important role in humans and other animals and act as an immunomodulator. LAB are helpful in disease treatment and prevention, as well as for improved digestion and absorption of nutrients. Probiotic microorganisms include LAB i.e., *Lactobacillus acidophilus*, *L. bulgaricus*, *L. casei*, *L. plantarum*, *L. rhamnosus*, etc. Use of these live bacteria to elicit an immune response or to carry a vaccine component is a new development in vaccine formulation. The advantages of live bacterial vaccines are their ability to mimic the natural infection, their intrinsic adjuvant properties, and that they can be administered orally. Components of pathogenic and nonpathogenic food-related microbes are currently being evaluated as candidates for oral vaccines.

4.1 Introduction

Live vaccines have played an important role since the beginning of vaccinology. Within the last two decades, the concept of live vaccines has gained substantial interest due to our increased immunological understanding and the availability of various techniques making the construction of safer live vaccines (Lindberg 1995).

The term "probiotics" was originally used by Lilley and Stillwell (1965) to indicate a substance that stimulates the growth of other microbes. The meaning of this

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_4,

term has recently been modified and is restricted to "a viable microbial agent which, when used for other organisms such as animal or man, beneficially affects the host by improving the balance of the normal microflora" (Fuller 1991). The first report regarding beneficial effects of probiotics was carried out by Metchnikoff in the early 1990s (Salimen et al. 1996) in which the useful effects of fermented milk in humans were documented. That consuming live microbes (such as lactic acid bacteria, LAB) in sour milk may help to improve the balance of the intestinal microflora was also reported.

Probiotic bacteria must survive in the oral cavity and be resistant to pH, bile acid, proteolytic enzymes, antimicrobial peptides, and intestinal peristalsis. Pathogenic and nonpathogenic food-related bacteria are currently being evaluated as live vaccines (Metchnikoff 1908).

Lindberg (1995) has conducted an excellent review of the history of live bacterial vaccines (Kajikawa et al. 2007). The first live bacterial vaccine was used in Spain in 1884 and involved a subcutaneous injection of weakened *Vibrio cholerae* (Detme and Glenting 2006). That study was followed by field trials in India with a more effective *V. cholerae* as a vaccine through a parenteral route (Table 4.1).

4.2 Probiotics as Antibiotics or Lactobiotics

Probiotic microorganisms are innocuous and are indicated as GRAS (generally regarded as safe) status in animal and human systems (Underdahl et al. 1982). Safety evaluation of a probiotic strain(s) includes (1) the ability of cells to produce secondary metabolites (e.g., sakacin, salivaricin, enterocin, formic acid, diacetyl, hydrogen peroxide, and acetoin) and enzymes (α-galactosidase, α-glucosidase, nitroreductase, β-glucosidase, etc.); (2) adhesion properties (e.g., α-enolase) that help them to adhere to the epithelium; (3) factors that influence the strain survival; and (4) interactions with the host body, particularly in terms of prevention of pathogenic microbes (Chukeatirote 2003). LAB are Gram-positive bacteria consisting of various genera including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Bifidobacterium*, and *Weissela*. *Lactobacillus* is the most widely used for probiotics (Havenaar et al. 1992; Greene and Klaenhammer

Table 4.1 Microbial probiotics and their safety status

Organisms	Infection potential
Lactobacillus	Mainly nonpathogens; a few opportunists reported in AIDS patients
Lactococcus	Mainly nonpathogens
Streptococcus	Opportunists; only *S. thermophilus* is used in dairy products
Enterococcus	Opportunists; some strains exhibit antibiotic resistance
Bacillus	Only *B. subtilis*, GRAS status, is reported in probiotics use
Bifidobacterium	Mainly nonpathogens; some strains are isolated from human infection
Propionibacterium	Dairy propionibacterial group is a potential candidate for probiotics
Saccharomyces	Mainly nonpathogens; some strains are isolated from human infection

Source: Adapted from Donohue and Salminen (1996)

Table 4.2 Probiotic bacteria and their effects

Strain	Beneficial effect
Lactobacillus acidophilus LA1	Adherence to human intestinal cells
	Balances intestinal microflora
	Immune enhancement
Lactobacillus GG	Prevention of antibiotic-associated diarrhea
	Treatment of rotavirus diarrhea
	Treatment of diarrhea caused by *Clostridium difficile*
	Stabilization of Crohn's disease
Lactobacillus casei Shirota	Prevention of intestinal microbiota disturbance
	Positive effects on bladder cancer
Lactobacillus gasser	Carcinogenic-associated enzyme reduction
Bifidobacterium bifidum	Prevention of viral diarrhea
Propionibacterium freudenreichii	Growth stimulation of other "friendly" bacteria

Source: For detailed references, see Salimen et al. (1996)

1994; Reid 1999; Reid et al. 1993; Bernet et al. 1994; Sarem-Damerdjii et al. 1995; Kirjavainen et al. 1998; Ouwehand et al. 1999; Casas and Dobrogosz 1997; Holzapfel et al. 1998; Netherwood et al. 1999; Jack et al. 1994; Reid and Burton 2002; Gonzalez et al. 1995; Khansari et al. 1990) (Table 4.2).

4.3 LAB as an Immune Enhancer

Consumption of *Lactobacillus* as a probiotic has been suggested for its disease resistance benefits including immune system enhancement (IL-6 and IL-10 production) and resistance to malignant growth (α-TNF production) and other infectious diseases. Malfunctioning of the immune system in aging, stress, and from infectious diseases (e.g., AIDS) and undernourishment are well established (Goodwin 1995; Pawelec et al. 1995; Woodward 1998; Lin and Chen 2000).

The above deficiencies can be overcome by immunomodulation with the aid of suitable natural and chemical agents and/or products. Currently available immunostimulatory products are often associated with deleterious side effects, however (Lin et al. 1989; Tahri et al. 1996). The development of natural products with immunomodulatory properties while being devoid of side effects, would therefore be important to improving the health of large populations with impaired immune function (Dietrich et al. 2003).

Vaccination is an efficient and cost-effective means for preventing infectious diseases, but available vaccines are delivered by injection, which has made mass immunization expensive and hazardous. Oral vaccines have several merits when compared with parenteral vaccines, but their used has been limited against mucosally transmitted pathogens (Benyacoub et al. 1999). Their potential for controlling nonmucosally transmitted diseases has not been accepted.

The development of oral live vaccines against pathogens is a major challenge in combating infectious diseases. The simple administration by mouth into the host is an attractive alternative to long-term drug treatments in a large population with

limited health care resources. The use of bacteria to induce an immune response to itself or its component as vaccine is an attractive vaccine strategy (Lindberg 1995). Advantages of live vaccines include (1) they mimic the pathogen or antigen; (2) they possess adjuvant properties; and (3) they can be administered by the oral route (Kotloff et al. 1996). Derivatives of pathogenic and nonpathogenic food-related bacteria are also used as a source of oral vaccines (Donohue and Salminen 1996; Lindberg 1998).

Live attenuated bacteria as an oral vaccine is not a new concept (Kochi et al. 2003). Previously attenuated *Salmonella enterica* serovar of Typhi administered as an acid-resistant capsule has already been marketed for use as a vaccine against typhoid. Attenuated *V. cholerae* forms the basis of a marketed vaccine against cholera. However, there is a possibility of using attenuated bacteria as a vehicle for delivering heterogonous antigens, i.e., antigens against pathogens other than themselves.

Derivatives of both pathogenic and nonpathogenic bacteria can be used as live vaccines; these include *Salmonella typhi*, *Shigella flexneri*, *Listeria monocytogenes*, *V. cholerae*, and *Escherichia coli* as well as some nonpathogenic bacteria such as *Lactobacillus* and *Bifidobacterium* (McGhee et al. 1992; Nardelli-Haefliger et al. 1996; Klijn et al. 1995).

These probiotic bacteria targets inductive sites of the host immune system such as mucosal surfaces and antigen presenting cells (APC) on macrophages. Use of LAB for the delivery of the vaccine is less exploited than is for attenuated pathogens (Fuller 1989). These are safe and the availability of genetic tools for recombinant gene expression in LAB is attractive for use as a vaccine candidate. LAB are not pathogenic and the vaccine delivery to APC may be less effective than for invasive bacteria. Regardless, however, specific immune responses have been obtained with several LAB. Some health benefits claimed for probiotics include improvement in the maintenance of indigenous microflora, prevention of infectious diseases and allergies, decrease in serum cholesterol levels, anticancer activity, stabilization of the gut mucosal barrier, immune adjuvant properties, alleviation of intestinal bowel disease symptoms, and improvement in the digestion of lactose in intolerant hosts (Strobel 1995).

4.4 Probiotics and GALT Immunity

The functioning of the gut mucosal immune system requires a complex network of signals with multiple interactions between commensal and foreign antigens and with the host cell. These host cells include epithelial cells, macrophages, dendritic cells, and other cells that belong to the nonspecific barriers mucous-producing cells such as goblet cells, and Paneth cells, which secrete antimicrobial peptides and produce cryptidins or defensins (Phillips-Quagliata and Lamm 1988).

Nonpathogenic probiotic bacteria must interact with the epithelial cells and GALT (gut-associated lymphoid tissue) (Fig. 4.1). The mucosal epithelial cells are

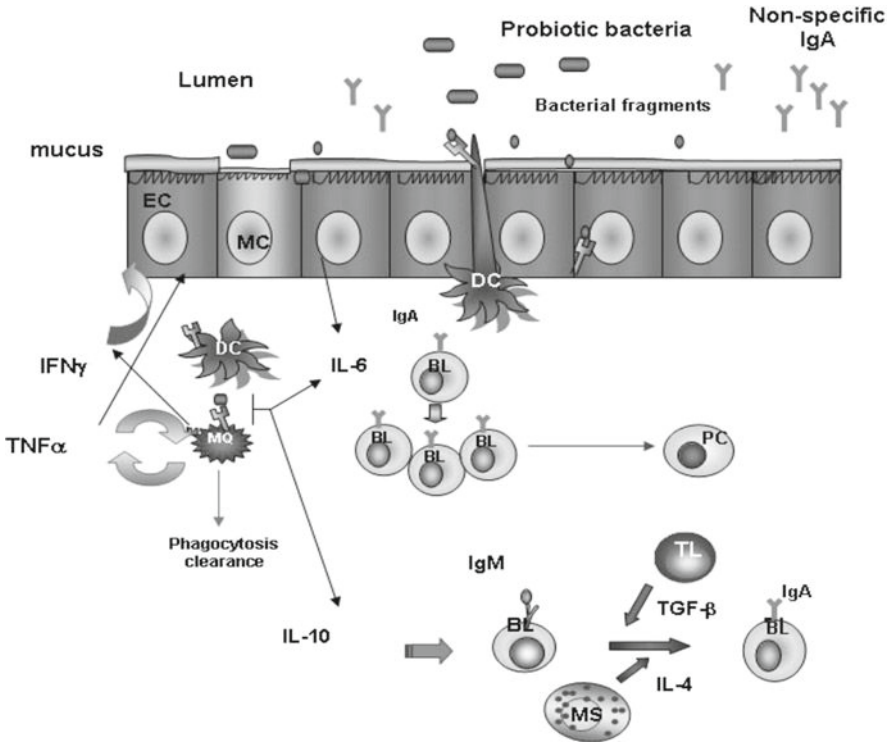

Fig. 4.1 The local immune response in the gut induced by the interaction between probiotic bacteria and the epithelial and immune cells associated with the lamina propria of the small intestine

important because they coordinate defense mechanisms by releasing chemokines and cytokines (IL-2, IL-10, and IL-6) that check the immune cells in both the specific and the nonspecific immune responses. This response must not be triggered by harmless intestinal commensal bacteria, and the inflammatory response must be controlled. Probiotic bacteria produce luminal secretory IgA (Link-Amster et al. 1994).

The increase in the number of IgA-producing cells was the most remarkable property induced by probiotic microorganisms and by fermented milk yogurt (Reference). The physiological role of IgA in the mucosal surface is unquestionable. Some probiotic bacteria can act as adjuvants of the mucosal and systemic immune response (Perdigón and Alvarez 1992).

Probiotic microorganisms are capable of inducing a gut mucosal immune response, which requires bacteria to interact with the epithelial and immune cells in the gut to induce the network of signals involved in an immune response. In the gastrointestinal tract, LAB correspond with the various pathways to interact with antigens (Neutra and Krahenbuhl 1996). These bacteria (as whole cells or as antigenic fragments) interact with the M cells in the Peyer's patches, with gut epithelial

cells, and with the associated immune cells. Cells release cytokines that induce up- or downregulation of the immune response (Mattingly and Waksman 1978). Bacterial vaccine vectors induce the production of multiple cytokines, including tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and interleukin-12 (IL-12), and proinflammatory mediators such as reactive nitric oxide (RNO), which enhance early innate immunity and create a local environment favorable for antigen presentation.

Mucosal epithelial cells form an efficient barrier, which prevents antigens of environmental pathogens gaining access to the host mucosal immunity and is responsible for more than two-thirds of the activity of the entire immune system. These findings are based on considerations of the numbers of immunecompetent cells, extent of the mucosal tissues, and quantities of immunoglobulins produced at these sites. Mucosal immunity is distinguished from systemic immunity by the abundance of secretory immunoglobulin i.e., s(IgA) and dedicated cellular flagellated microorganisms including commensals. These entities trigger epithelial homeostatic chemokine responses that recruit immune cells of the innate immune system to the gut epithelium and lamina propria of the intestinal mucosa to link between the humoral and cell-mediated immune response.

Recognition to TLR (Toll-like receptor) by LAB can activate the signal of TLRs, mainly as TLR 2 and TLR 4. These peptides are the dominant receptor of lipopolysaccharides of bacterial species. TLR 2 mediates signals from other bacterial components including lipoteichoic acid (LTA), peptidoglycan, and lipoproteins and/or lipopeptides (Singh et al. 2008). However, the exact location of these receptors in the intestinal epithelial cells remains controversial. TLR signals are essential, not only for response against pathogens, but also to maintain intestinal barrier functions. For an effective application as an immune enhancer, there should be multiple consequences of the cross talk between the probiotic bacteria and intestinal mucosa. Probiotic bacteria may impart beneficial effects through colonization and/or release of some bioactive compounds (e.g., enzymes, peptides, and bacteriocins). These functions translate into reinforcement of the intestinal barrier as well as direct modulation of epithelial cell functions including cytokine and chemokine release (IL-6, IL10, IL-2, IL-8, and TGF-β). This elicits innate and adaptive immunity and production of cytokines by monocytes/macrophages. The result is an increase in the signals to the epithelial cells and other immune cells and provision of microbial antigens to native T cells in the Peyer's patches and mesenteric lymph nodes (MLN). This activates an IgA antibody-mediated mucosal response to check the bacterium to prevent overgrowth and spread beyond MLN (Weissman et al. 2000).

There is evidence for the uptake of nonpathogenic bacteria or their fragments by macrophages or dendritic cells in the lamina propria, which is possible through direct sampling of luminal antigen for dendritic cells through TLRs and CD-206 mannose receptors (Akira et al. 2001). These bacteria can be cleared or transported to the mesenteric lymphatic node in which they interact with T and B cells to induce specific mucosal IgA or suppress T cells (Th3, Tr1, and $CD25^{+}$) (Holder and Freeman 1981) (Fig. 4.2).

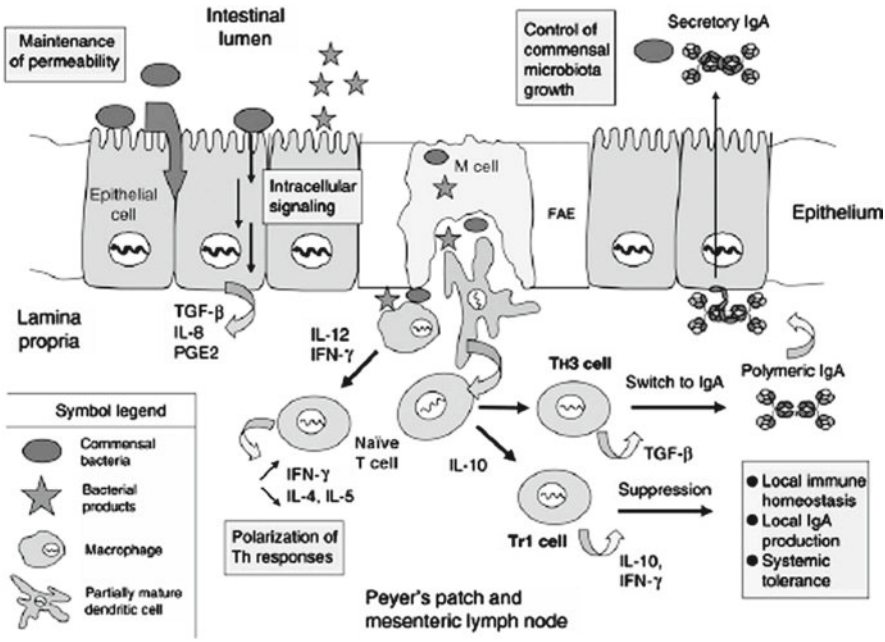

Fig. 4.2 Schematic diagram showing cross talk between the probiotic bacteria and the intestinal mucosa

4.5 The Demise of the Needle

Probiotic bacteria have long been considered useful because of the changes which they induce in the intestinal flora. Oral vaccines from probiotic strains of *Lactobacillus* are active against many diseases caused by bacteria, viruses, and protozoa.

4.5.1 Malaria

Merozoite surface protein 1 (MSP1) is a common surface protein layer which is found in most *Plasmodium* species. MSP1 has been studied as an important candidate for a vaccine against malaria (Blackman et al. 1991). High-level expression of MSP1 by *Plasmodium* in the asexual stage plays an important role in its entry in RBCs. MSP1 can be proteolytically cleaved into five fragments. It is a two step process. Matured merozoite contains carboxyl-terminal 19-ku fragment (MSP-119) on its surface (Miller et al. 1993).

These protective antigens can be delivered to the mucosal surfaces using live bacteria containing plasmids responsible for the expression of a specific antigen.

These antigens are derived from attenuated pathogenic microorganisms such as *S. typhi* and *Chlorella*. As an alternative, harmless food-grade bacteria such as LAB are being evaluated for their efficacy as a live antigen candidate. LAB can survive in the gastrointestinal tract of humans and other animals with a retention time of 2–3 days; it does not destroy or colonize the mucous membranes, and yet does not elicit strong host immune responses. The immunogenicity of soluble protein is low when administered orally, but by using genetically engineered bacteria immunogenicity can be enhanced, so the low-level expression of MSP-119 in *L. lactis* is still able to elicit strong protection against malarial parasites (Mercenier et al. 2000).

4.5.2 AIDS

AIDS is a deadly disease alternatively, we can consider it to be a collection of diseases. Safe, powerful, and cost-effective vaccines, which can induce both mucosal and systemic immunity, may be required to limit the spread of the HIV virus. In the past few years, multiple strategies to produce an immunogenic HIV vaccine have been discovered (Ke-Qin et al. 2003). This includes HIV subunit peptide vaccines, DNA as a vaccine, recombinant virus-vector vaccines (using viral vectors such as Vaccinia virus, Adenovirus, Rabies virus, Flavivirus, Friend murine leukemia virus, Venezuelan equine encephalitis virus, and adeno-associated virus), and bacteria as a vector (e.g., Bacille Calmette-Guerin). Each of these strategies has shown promise in animal models (Schnell et al. 2000); they can be used alone or in combination. However, there is still a need for a safe and highly effective HIV vaccine. In a study conducted in mice, it was demonstrated that oral administration of recombinant *L. lactis* encoding the V2–V4 loop of the HIV *env* gene can induce HIV-specific mucosal and systemic immunity. Induced humoral and cell-mediated immune response is sufficient to impart immunity against an HIV *Env* – expressing vaccinia virus challenge in mice (Aldovini and Young 1991).

Oral administration of recombinant *L. lactis*-associated vaccine that contains the V2–V4 loop of the HIV virus can protect against AIDS and also significantly reduce viral load. These findings make recombinant *L. lactis* an appropriate candidate for HIV vaccine development.

4.5.3 Infantile Diarrhea

Rotavirus is the major cause for severe infantile diarrhea, responsible for over two million diarrheal episodes worldwide. The first rotavirus vaccine was the Rota Shield, first used in the USA. Its efficacy was rated as high as 80–100%; however, in 1999 the US Centers for Disease Control and Prevention (CDC) found an association between Rotashield and a potentially fatal bowel obstruction called intussusceptions. As a result, Rotshield has been withdrawn from the market since 1999. In 2004, a new vaccine called “Rortarix” came into existence and is determined to be 70% efficient

(Reference). Rotarix was found to cause side effects such as incidence of low-grade fever, so it was not popularized. Another vaccine, RotaTeq, is a live attenuated vaccine in which passively produced antibodies provide protection against rotavirus (Anderson and Weber 2004). Over the last few years, the role of probiotics, especially *Lactobacillus* species preventing rotavirus diarrhea, has been recognized. A combination of species of *Lactobacilli* with anti-rotavirus antibodies produced in animals was studied in mouse pups along with rotavirus (Velazquez et al. 1996). *L. rhamnosus* GG, a well-known probiotic, was found to synergize with antiviral antibodies and helped in early recovery of diarrhea in mice and saved up to 90% of antibodies. *L. casei* (Strain GG) isolated from human samples, now popularly known as *L. rhamnosus*, has been used in Finland for recovery from acute rotavirus diarrhea in children (Gomboova et al. 1986). This treatment is administered in the form of a powdered fermented milk product. In children with rotavirus diarrhea, recovery occurred within 1.5 days in infants treated with *Lactobacillus reuteri* versus 2.5 days in the matched control infants (Korik et al. 1968). These organisms also reinforce local immune defenses through specific IgA response to rotavirus.

4.5.4 Trichomoniasis

This disease, commonly known as urinary tract infection or urinogenital tract infection, is asexually transmitted. An immunotherapeutic effect on trichomoniasis is accomplished by stimulation of the humoral/B-cell immune response in the serum and probably also in the cervical secretion of the host (Tatyana et al. 2000). Until 1959, topical vaginal preparations were available against trichomoniasis which had provided relief but were ineffective as cures. Azomycin was used for the treatment of trichomoniasis, marketed as "Flagyl." Azomycin was ineffective against *T. vaginalis*. Solco Trichovac is the only commercially available vaccine. The mode of action of the bacterial vaccines prepared for trichomoniasis is not satisfactory, however; the vaccine composed of the LAB induce cross-reacting antibodies against abnormal *Lactobacillus* and *T. vaginalis* without adversely affecting the growth of normal *Lactobacilli* in the vagina. This concept of antigenic similarity of two such unrelated and serologically variable groups of organisms as *Lactobacilli* and *Trichomonads* is rather surprisingly it resides in the cervical secretion of the host (Classen et al. 1995). This mode of action of induction of antibodies against aberrant *L. acidophilus*, which cross-react with *T. vaginalis* but not with LAB, is a new concept for a vaccine candidate.

4.5.5 Ischemic Heart Diseases

Protection of the heart and prevention of ischemia is a difficult and multifaceted phenomenon with potential clinical applications. Previous reports suggest that heat shock proteins (HSPs) and proteins with antioxidative activity are helpful in

preventing myocardial infarction. Hormone-mediated signaling mechanisms may also be involved in preventive treatment. Myocardial tolerance to a subsequent challenge against ischemia and reperfusion by sublethal doses of Gram-negative bacterial endotoxin (lipopolysaccharides; LPS) is well known (Morimoto and Santoro 1998); however, the toxic nature of the endotoxin has precluded its clinical application. Use of an endotoxin analog with reduced toxicity, the 48-monophosphoryl derivative of lipid A (MLA), has opened up new possibilities for studying protective mechanisms (Kukreja et al. 1996). Despite the reduced toxicity of MLA, such agents are administered in limited doses due to their toxic effects on the host. A *Lactobacillus* preparation is used as a nontoxic bacterial-derived agent for the formulation of new drugs that have no toxic effects (Goldin et al. 1992). Increased myocardial tolerance to ischemia–reperfusion damage, similar to that demonstrated with Gram-negative bacteria (endotoxin), might be obtained by using different bacterial strains capable of enhancing nonspecific resistance (Elmer 2001). *Lactobacilli* are generally recognized as safe organisms based on the fact that they are devoid of LPS and lipid A in their cell walls, and also because they stimulate the host's nonspecific immunity (adjuvanticity).

4.5.6 Gastritis, Peptic Ulcer, and Gastric Adenocarcinoma

Helicobacter pylori is recognized as a human-specific gastric pathogen that colonizes the stomach of at least half the world's population. Most infected individuals seldom show any symptoms (Matsuzak and Chin 2000). Infection is associated with the formation of duodenal and gastric ulcers, inflammation of the colon and tumors. Prevention of adherence of the pathogen and its colonization at mucosal surfaces is best achieved when immunity is enhanced after local stimulation. Different delivery systems satisfying this requirement are currently under development, with various avenues being explored for oral administration (Isolauri et al. 1991). One such delivery system is based on live bacterial vectors including nonpathogenic, noninvasive LAB strains (Shornikova et al. 1997). These do not induce pronounced proinflammatory responses, which renders them best suited for immunocompromised subjects, infants, and elderly individuals.

4.6 Conclusion/Future Recommendations

The rationale behind the development of dietary LAB as a live mucosal delivery system is that they have been used from time immemorial in the preparation of fermented foods and feeds and have thus been consumed worldwide by humans and animals. Moreover, specific LAB strains have been shown to exert beneficial health effects, i.e., probiotic effects and to be particularly adapted to immunization by the oral route, since they are quite acid resistant.

Administering vaccines orally, for example, through drinking water, provides excellent disease protection while eliminating the problems associated with parenteral injections. Oral vaccination offers numerous benefits including convenience and efficiency in treating very large numbers of patients. Oral vaccines are safe, noninvasive, nonpathogenic, and have good adherent properties. *Lactobacillus* is a noble oral vaccine candidate. LAB is capable of delivering antigens to the mucosal and systemic immune systems thus eliciting specific antibody responses in serum and secretions. Notably, this carrier seems to induce a mixed Th1/Th2-type immune response.

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Chapter 5
Microbially Synthesized Nanoparticles: Scope and Applications

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Abstract The critical need for development of reliable and eco-friendly processes for synthesis of metallic nanoparticles has recently been realized in the field of nanotechnology. Increasing awareness toward green chemistry and biological processes has elicited a desire to explore environmentally friendly approaches for the synthesis of nanoparticles as a safer alternative to physical and chemical methods, which involves harsh conditions and use of hazardous chemicals. Therefore, the use of natural resources, including bacteria and fungi, has been exploited for cost-effective and environmentally nonhazardous nanoparticle synthesis. The rich microbial diversity of bacteria and fungi contains the innate potential for the synthesis of nanoparticles and may be regarded as potential biofactories. In fact, microbial synthesis of nanoparticles has emerged as an important branch of nanobiotechnology. The synthesis of inorganic materials by biological systems occurs through remarkable processes at ambient temperature and pressures and neutral pH. Among the various biological systems, bacteria are relatively easy to manipulate genetically, whereas fungi have an advantage of easy handling during downstream processing and large-scale production. In spite of the successes achieved in biological synthesis of nanoparticles, there is still a need to improve the rate of synthesis and monodispersity of nanoparticles. Also, microbial cultivation and downstream processing techniques must be improved, and more efficient methods should be developed. Furthermore, in order to exploit the system to its maximum potential, it is essential to understand the biochemical and molecular mechanisms involved in nanoparticle synthesis. Delineation of specific genomic pathways and characterization of gene products involved in biosynthesis of nanoparticles are required. The underlying molecular mechanisms that mediate microbial synthesis of nanoparticles will help in understanding the molecular switches and factors necessary to control the size and shape, as well as crystallinity of nanoparticles. Indeed, biological systems are still relatively unexplored, and therefore, the opportunities are

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_5,

open for budding nanobiotechnologists to utilize nonpathogenic biological systems for metallic nanoparticle synthesis with commercial perspectives.

5.1 Introduction

Nanotechnology is a fascinating area that is emerging as a cutting-edge technology encompassing interdisciplinary subjects such as physics, material science, chemistry, biology, and medicine. The prefix "nano" in the term nanotechnology is derived from a Greek word *nanos*, which means "dwarf". It refers to any engineered matter that is one billionth (10^{-9} m) in size and expressed as nanometer (nm) or roughly the length of three atoms side by side. Comparative analysis of nanoparticle size with that of other molecules indicates that the DNA molecule is 2.5 nm wide, a protein is approximately 50 nm in length, and a flu virus is about 100 nm vis-à-vis a human hair, which is approximately 10,000 nm thick. The concept of nanotechnology was first presented by Richard Feynman in 1959 through his famous lecture, at the American Institute of Technology, entitled "There's plenty of room at the bottom". Nanotechnology is a multidisciplinary field that has attracted the attention of material scientists, mechanical and electronics engineers, medical researchers, biologists, physicists, and chemists. With advancements in nanoparticle synthesis, many new applications of nanomaterials are emerging rapidly. In fact, the synthesis of nanoparticles is regarded as a cornerstone of nanotechnology. Developing new methods for nanoparticle synthesis is an active research area. The surge of interest in this field is due to the distinctness of nanoparticles in their physical, chemical, electronic, electrical, mechanical, magnetic, thermal, dielectric, optical, and biological properties as compared with the characteristics of bulk materials (Schmid 1992; Daniel and Astruc 2004).

Diminution of particle size exerts pronounced effects on the physical properties of nanoparticles. The change in their physical properties is due to the large surface area, large surface energy, spatial confinement, and reduced imperfections. Nanoparticles are significantly different from bulk materials owing to their surface plasmon resonance, enhanced Rayleigh scattering, surface enhanced Raman scattering, quantum size effect, and supermagnetism. Therefore, they serve as basic units for next-generation electronics, optoelectronics, and a range of chemical and biochemical sensors, based on their size, shape, and crystallinity (Ramanavicius et al. 2005). Typically, nanoparticles measure 0.1–100 nm in each spatial dimension and are commonly synthesized using top-down and bottom-up strategies (Fendler 1998). In the top-down approach, the bulk materials are gradually broken down to nano-sized materials by machining and etching techniques. In contrast, the atoms or molecules are assembled into molecular structures in the nanometer range in the bottom-up approach, which is commonly applied for chemical and biological synthesis of nanoparticles.

Generally, the methods used for nanoparticle synthesis follow chemical routes that are not environmentally friendly and often generate hazardous by-products,

which could potentially pollute the environment. Chemical synthesis involves conditions such as high temperature, high pressure, and environmental inertness; such synthesis reactions are also cost-intensive (Rao et al. 2003). Furthermore, the use of toxic chemicals and organic solvents during nanoparticle synthesis and their occurrence on the surface of nanoparticles limit their applications. Such drawbacks necessitate the development of clean, biocompatible, nonhazardous, and eco-friendly methods for nanoparticle synthesis. Consequently, biological systems have been focused on and exploited as a preferred, green alternative for synthesis of nanoparticles. In nature, living organisms from bacteria to beetles rely on protein-based nanomachines, which perform excellent jobs from whipping flagella to flexing muscles. Indeed, the molecular machinery evolved by nature surpasses everything that mankind knows and designs with conventional manufacturing technology (Lowe 2000). Undoubtedly, biological systems have a unique ability to control the structure, phase, orientation, and nanostructural topography of inorganic crystals (Cui and Gao 2003).

It is well known that microbes such as bacteria (Beveridge and Murray 1980; Brierley 1990), yeast (Huang et al. 1990), fungi (Frilis and Myers-Keith 1986), and algae (Sakaguchi et al. 1979; Darnall et al. 1986) are capable of adsorbing and accumulating metals. These microorganisms could be used for recovery of metals and reduction of environmental pollution (Klaus et al. 1999; Sharma et al. 2000; Mukherjee et al. 2001a; Nair and Pradeep 2002; Oremland et al. 2004). The potential of microbes to reduce metals has provided another new dimension of "Quantum Dots" or bimetallic nanoparticles with immense use in semiconductor devices (Dameron et al. 1989). A well-known example of reduction of metals includes the magnetotactic bacteria that synthesize magnetic nanoparticles (Schuler and Frankel 1999) with widespread applications (Safarik and Safarikova 2002). Also, lactic acid bacteria in whey of buttermilk exhibit the capability of producing gold–silver composite materials when challenged with a mixture of the two metal ions. Fungi, due to their tolerance to metals and metal bioaccumulation ability, are well-suited for metal nanoparticle generation (Sastry et al. 2003). Based on their enormous biotechnological applications, microorganisms such as bacteria, fungi, and yeast are now regarded as possible eco-friendly "nano-factories" (Ahmad et al. 2002).

Microbial resistance to toxic heavy metals is due to the chemical detoxification and energy-dependent ion efflux from the cell by membrane proteins that function as either ATPase or chemiosmotic cation or proton anti-transporters. The detoxification of metal ions occurs by reduction and/or precipitation of soluble toxic inorganic ions to insoluble nontoxic metal nanoclusters. Such processes could be accomplished by either extracellular biomineralization, biosorption, complexation, precipitation or intracellular bioaccumulation. Microbes produce the inorganic materials either intra- or extracellularly in nanoscale dimensions. In the case of intracellular production, the accumulated particles are of relatively smaller dimension with low polydispersity. Since polydispersity is a major concern for practical commercial nanoparticle synthesis, it is important to optimize the conditions for monodispersity in biological processes (Bao et al. 2003). For controlling the size and shape of biological nanoparticles, genetically engineered microbes capable of

producing specific reducing agents can be developed. Nevertheless, the combinatorial approach such as photobiological methods, as demonstrated in the case of *Fusarium oxysporum*-mediated silver nanoparticle production (Mohammadian et al. 2007), could be helpful in increasing the rate of production. Moreover, there are certain advantages to fungal synthesis of nanoparticles such as (1) economic viability, (2) ease in scale-up as in the thin solid substrate fermentation method, (3) ease in handling biomass, and (4) large-scale secretion of extracellular enzymes. Although biological methods are regarded as safe, cost-effective, sustainable, and environmentally friendly, they still have some drawbacks in terms of culturing of microbes, which is time-consuming and difficult in providing optimal control over nanoparticle size distribution, shape, and crystallinity. However, proper strain selection and optimization of conditions such as pH, incubation temperature and time, concentration of metal ions, and amount of biological material can help in successful implementation of biological and biomimetic approaches for large-scale nanoparticle production for commercial applications.

5.2 Nanoparticle Synthesis by Bacteria

Bacteria are among the most extensively exploited natural resources for synthesis of metallic nanoparticles. The key reason for bacterial preference for nanoparticle synthesis is their relative ease of manipulation. While exploring the secrets of microbial synthesis of nanoparticles, the formation of magnetite particles was documented in magnetotactic bacteria (Lovley et al. 1987; Dickson 1999), siliceous materials by diatoms (Pum and Sleytr 1999), and gypsum and calcium layers by S-layer bacteria (Milligan and Morel 2002). The interactions between metals and microbes have been exploited for various biological applications in the fields of bioremediation, biomineralization, bioleaching, and biocorrosion (Klaus-Joerger et al. 2001). Lately, the microbial synthesis of nanoparticles has emerged as a promising field of research as has nanobiotechnology. The status of research on biosynthesis of some generally studied and commonly used metal nanoparticles by different bacteria, actinomycetes, and cyanobacteria is discussed below.

5.2.1 Silver Nanoparticles

A plethora of methods, namely, photocatalytic reduction (Chang et al. 2006), chemical reduction (Yu 2007), radiation–chemical reduction, metallic wire explosion, sonochemistry, polyol process (Nersisyan et al. 2003), photoreduction (Courrol et al. 2007), reverse micelle-based methods (Xie et al. 2006), matrix chemistry (Ayyad et al. 2010), and biological synthesis (Zeiri et al. 2002; Shahverdi et al. 2007; Durán et al. 2007; Sathishkumar et al. 2009; Kalishwaralal et al. 2010) have been employed for production of silver nanoparticles. Klaus et al. (1999)

reported generation of silver crystals using silver-resistant bacterium *Pseudomonas stutzeri* AG259 isolated from silver mines. This bacterium has been found to generate pyramidal and hexagonal silver nanoparticles measuring up to 200 nm in size, embedded in the organic matrix of the bacterial cell. Similarly, *Morganella* sp. RP-42, an insect midgut isolate, upon exposure to silver nitrate ($AgNO_3$), produced extracellular crystalline nanoparticles measuring 20 ± 5 nm. Three gene homologues (*sil*E, *sil*P, and *sil*S) have been identified in silver-resistant *Morganella* sp. The homologue of *sil*E from *Morganella* sp. showed 99% nucleotide sequence similarity with the previously reported gene, *sil*E, encoding a periplasmic silver-binding protein. Also, the cells of *Corynebacterium sp.* SH09 have been shown to produce silver nanoparticles at 60°C within 72 h on the cell wall in the size range of 10–15 nm with diamine silver complex $[Ag(NH_3)^2]^+$ (Zhang et al. 2005). The silver-binding proteins provide amino acid moieties that serve as nucleation sites for the formation of silver nanoparticles. Silver precipitating peptides (AG3 and AG4) have also been found with the capability of precipitating silver from aqueous solution of silver ions and form face-centered cubic (fcc) structured silver crystals (Naik et al. 2002). Under normal conditions, the small periplasmic silver-binding proteins bind silver at the cell surface and by efflux pumps propels the incoming metals and protects the cytoplasm from metal toxicity (Li et al. 1997; Gupta and Silver 1998). An airborne *Bacillus sp.* reduced Ag^+ ions to Ag^0 and accumulated metallic silver nanoparticles of 5–15 nm size in the periplasmic space of the cell (Pugazhenthiran et al. 2009). Silver nanoparticles of diameter 6.4 nm have also been produced by dried cells of *Aeromonas sp.* SH10, which reduced $[Ag(NH_3)^2]^+$ to Ag^0 within 4 h. These particles were monodispersed and uniform in size and remained stable for more than 6 months without aggregation and precipitation (Mouxing et al. 2006). Culture supernatants of *Enterobacteriaceae* (*Klebsiella pneumoniae, Escherichia coli,* and *Enterobacter cloacae*) also rapidly synthesize silver nanoparticles in sizes ranging from 28.2 to 122 nm with an average size of 52.5 nm by reducing Ag^+ to Ag^0. Addition of piperitone partially inhibited silver ion reduction, which suggested the involvement of nitroreductase enzymes in the reduction process (Shahverdi et al. 2007). Similarly, the culture supernatant of nonpathogenic bacterium *B. licheniformis* has been used for the extracellular synthesis of silver nanoparticles of ~50 nm size (Kalishwaralal et al. 2008). Barud et al. (2008) demonstrated the formation of homogeneous silver containing bacterial cellulose membranes obtained from hydrated membranes of *Acetobacter xylinum* cultures soaked on silver ion with triethanolamine (Ag^+-TAE) solution. Recently, Musarrat et al. (2010) have reported the biosynthesis of silver nanoparticles in the size range of 5–27 nm, produced by an industrially important fungal strain KSU-09, isolated from the roots of date palm (*Phoenix dactylifera*). It has been demonstrated that mycelia-free water extracts obtained from mycelia suspended in water for 72 h facilitated the production of stable, predominantly monodispersed, and spherical nanoparticles upon addition of 1 mM silver nitrate, as determined by UV–visible spectroscopy, XRD, AFM, and TEM (Figs. 5.1–5.4). The infrared spectrum revealed the presence of fungal proteins in the medium, plausibly responsible for nanoparticle stability (Fig. 5.5). Thus, bacteria from the

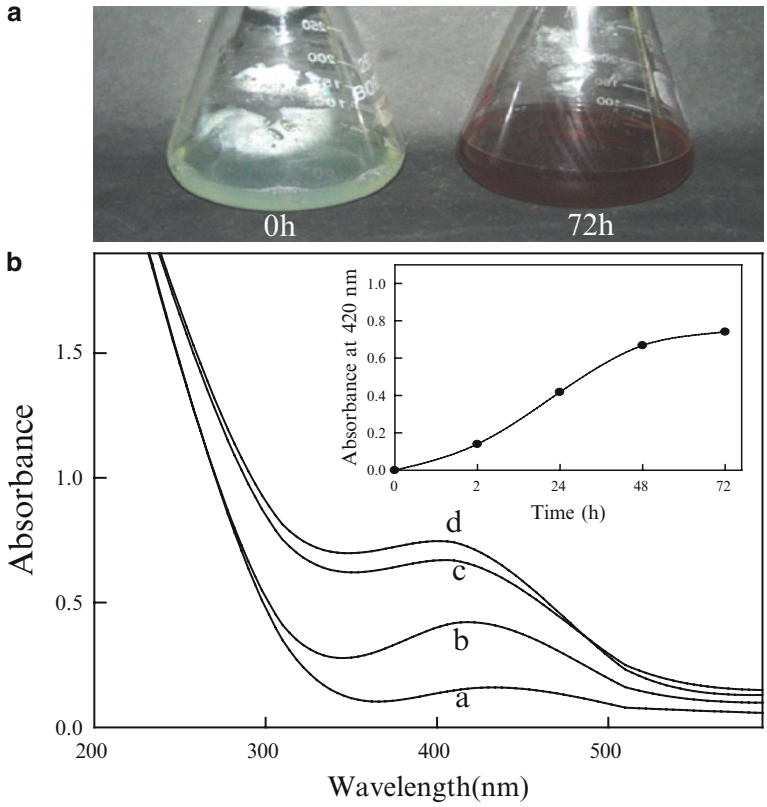

Fig. 5.1 Conical flasks containing fungal biomass in aqueous solution of 1×10^{-4} M $AgNO_3$ at the beginning and after 72 h of reaction. Panel (**b**) shows the UV–Visible absorption spectra of extracellularly synthesized silver nanoparticles at 420 nm exhibiting time-dependent increase in typical SPR bands upon (a) 2 h, (b) 24 h, (c) 48 h, (d) 72 h of incubation. The *inset* shows the change in SPR as a function of time (Adapted from Musarrat et al. 2010.)

environment could be exploited as a natural bioresource for simple, nonhazardous, and efficient synthesis of AgNPs for development of new generation nano-antimicrobials against multidrug-resistant microorganisms with a multitude of applications. This is discussed in a separate section of this chapter.

5.2.2 Gold Nanoparticles

Bacteria have been extensively used for the synthesis of gold nanoparticles. Ahmad et al. (2003a) demonstrated bacterial synthesis of monodispersed gold nanoparticles with extremophilic *Thermomonospora* sp. biomass via reduction of $AuCl_4$ ions through enzymatic processes. Konishi et al. (2004) reported gold

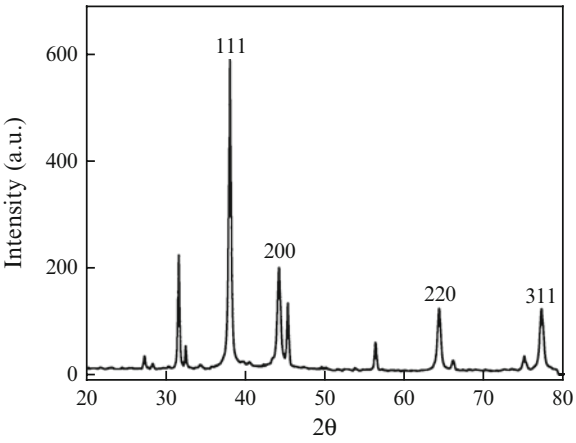

Fig. 5.2 XRD pattern depicting the crystalline nature of silver nanoparticles. Diffraction at 38.5°, 44°, 64.5°, and 72° 2θ indexed to the (111), (200), (220), and (311) planes of the face-centered cubic (fcc) silver, respectively. Particle size based on Scherrer's algorithm was 22 nm (Adapted from Musarrat et al. 2010.)

nanoparticle synthesis using the mesophilic bacterium *Shewanella*, with H_2 as an electron donor. Shiying et al. (2007) showed that the bacterium *Rhodopseudomonas capsulata* produced spherical gold nanoparticles in the range of 10–20 nm, upon incubation of bacterial biomass with aqueous chlorauric acid ($HAuCl_4$) solution at a pH range of 4.0–7.0. Solution pH is an important factor in controlling the morphology of biogenic gold particles and location of gold deposition in cells. Alkalotolerant *Rhodococcus* sp. produced more intracellular monodispersed gold nanoparticles on the cytoplasmic membrane than on the cell wall due to reduction of the metal ions by enzymes present in the cell wall and on the cytoplasmic membrane, but not in the cytosol (Ahmad et al. 2003b). Bacterial cell supernatants of *Pseudomonas aeruginosa* have been used for reduction of gold ions and for extracellular biosynthesis of gold nanoparticles (Husseiny et al. 2007). The exact mechanism leading to reduction of metal ions in organisms has not yet been elucidated. Nevertheless, gel electrophoresis observations revealed the presence of four different proteins ranging from 10 to 80 KDa, which could be responsible for reduction of the chloroaurate ions and capping of the gold nanoparticles. *Bacillus subtilis* 168 has been reported to reduce water-soluble Au^{3+} ions to Au^0 and produce nanoparticles of octahedral morphology and dimensions of 5–25 nm inside cell walls (Beveridge and Murray 1980). Heterotrophic sulfate-reducing bacterial enrichment from a gold mine has been exploited to destabilize gold (I)-thiosulfate complex $Au(S_2O_3)^2$ to elemental gold of 10 nm size in the bacterial envelope, releasing H_2S as an end product of metabolism (Lengke and Southam 2006). *E. coli* DH5α-mediated bioreduction of chloroauric acid to Au^0 resulted in accumulation of nanoparticles, mostly spherical and some triangles and quasi-hexagons, on the cell surface (Du et al. 2007). These cell-bound nanoparticles offer promising

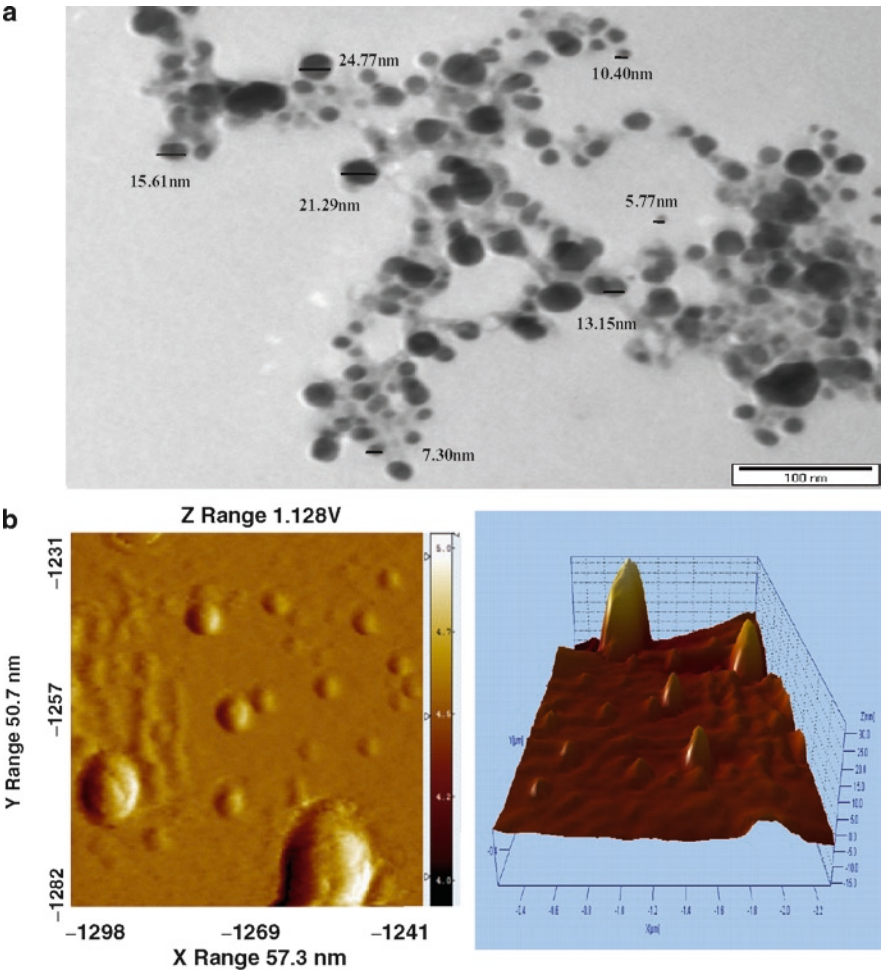

Fig. 5.3 The electron and atomic force microscopic analyses of AgNPs. Panel (**a**) shows the representative transmission electron micrograph recorded from a drop-coated film of the AgNPs produced by fungus on Morgagni™ 268 (d) instrument at a voltage of 80 kV. Panel (**b**) shows the 3D topography of nanoparticles in both the perspective and top views. Scan size is 5×5 μm. The intensity of color in side bar reflects the height of the particles (our unpublished data)

applications in electrochemistry of hemoglobin and other proteins (Du et al. 2007). Bioreduction of trivalent aurum has also been reported in the photosynthetic bacterium *Rhodobacter capsulatus*, which has a higher biosorption capacity of $HAuCl_4$ per gram dry weight in the logarithmic phase of growth. The carotenoids and NADPH-dependent enzymes embedded in the plasma membrane and/or secreted extracellularly have been found to be involved in the biosorption and bioreduction of Au^{3+} to Au^0 on the plasma membrane and also outside the cell (Feng et al. 2007).

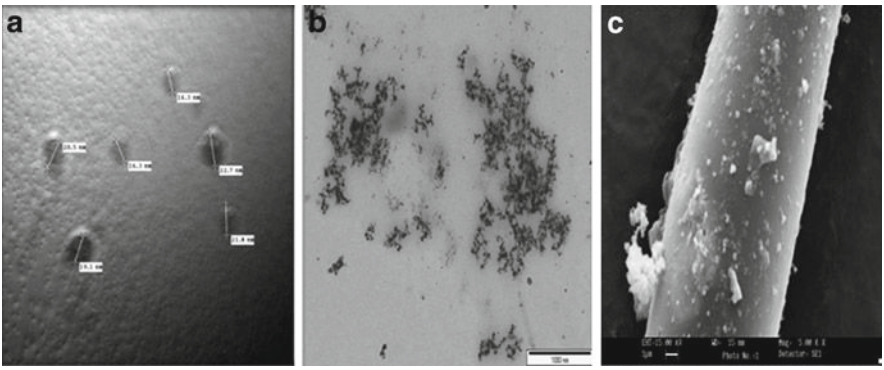

Fig. 5.4 Panel (**a**): Transmission electron-microscopic (TEM) image analysis of extracellularly produced silver nanoparticles in the size range of 16–23 nm, Panel (**b**): TEM image of intracellularly produced silver nanoparticles, Panel (**c**): Scanning electron-microscopic (SEM) image of fungus producing silver nanoparticles (our unpublished data)

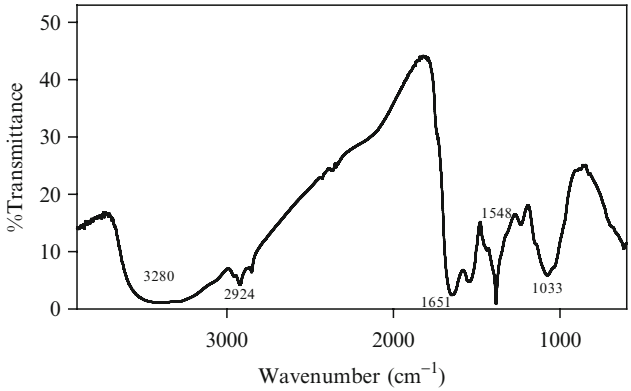

Fig. 5.5 FTIR spectrum of silver nanoparticles synthesized by fungus (our unpublished data)

5.2.3 Magnetic Nanoparticles

The synthesis of magnetic nanoparticles has been widely reported in magnetotactic bacteria, which are Gram-negative bacteria of diverse morphology and occur widely in marine and freshwater sediments. They are known to produce intracellular, membrane-bound magnetite (Blakemore et al. 1979), greigite, and pyrrhotite (Bazylinski et al. 1993). Mann et al. (1984) reported that a microaerophilic bacterium *Aquaspirillum magnetotacticum*, isolated from sediments, produces crystals of ordered single-domain magnetite (Fe_3O_4) particles with octahedral prism morphology of (111) faces truncated by (100) faces. The marine magnetotactic bacterium MV-1, isolated from sulfide-rich sediments of an estuarine salt marsh, anaerobically bioreduced nitrous oxide and ferric quinate to yield iron-rich magnetosomes.

Each magnetite (Fe_3O_4) particle is a parallelepiped with dimensions $40 \times 40 \times 60$ nm, with a single magnetic domain (Bazylinski et al. 1988). Similarly, magnetotactic bacteria isolated from brackish and marine sulfide-rich water and sediments intracellularly deposited single crystals of ferromagnetic iron sulfide, greigite (Fe_3S_4), reportedly associated with nonmagnetic iron pyrite (FeS_2) and aligned in chains. Each chain contains approximately ten nanoparticles measuring 75 nm in size. Most of the particles have irregular shape, whereas some exhibit octahedral and cubo-octahedral symmetry with strong diffraction contrast (Mann et al. 1990). Bacteria such as *Magnetospirillum magneticum* have demonstrated the ability to synthesize fine (50–100 nm) intracellular membrane-bound ferromagnetic particles composed of magnetite (Fe_3O_4) or greigite (Fe_3S_4). These particles are surrounded by an intracellular phospholipid membrane forming structures called magnetosomes (Schuler and Frankel 1999). Each bacterial cell contained from 0 to 45 nanoparticles with polydispersity. Magnetosomes comprise both crystallite and noncrystallite magnetic crystals. In *M. magnetotacticum* (MS-1), magnetite (Fe_3O_4) nanoparticles have been found assembled into single or multiple chains and anchored inside the cell, enabling the bacteria to passively orient themselves along the geomagnetic field. Each nanoparticle assembled in the bacterial phospholipid membrane has a cubo-octahedral crystal structure with a diameter of ~50 nm and magnetic moment of $\sim 6 \times 10^{-17}$ A m^2. Accumulation of magnetic iron mineral crystals into highly ordered chain-like structures was also evidenced in the magnetosomes of *M. gryphiswaldense* (Lang and Schuler 2006). Watson et al. (1999) demonstrated the synthesis of magnetic iron sulfide (FeS) nanoparticles of 2 nm on the surface of sulfate-reducing bacteria. Moreover, Bharde et al. (2005) studied magnetite nanoparticle synthesis by *Actinobacter*, a nonmagnetotactic bacterium. Lee et al. (2004) demonstrated that by manipulating magnetotactic bacteria in fluid using microelectromagnets, the assembly of magnetic nanoparticles inside the cell can be controlled. Furthermore, the multicellular magnetotactic bacterium *Candidatus Magnetoglobus multicellularis* has been reported to interact with the geomagnetic field on the basis of biomineralized magnetic nanocrystals (Perantoni et al. 2009). The magnetite nanoparticles formed by bacteria such as *A. magnetotacticum* (Mann et al. 1984), the magnetotactic bacterium MV-1 (Bazylinski et al. 1988), Sulfate-reducing bacteria (Watson et al. 1999), *M. magnetotacticum* (Lee et al. 2004), and *M. gryphiswaldense* (Lang and Schuler 2006) largely exhibited octahedral prism, parallelepipeds, cubo-octahedral, and hexagonal prism morphologies in the size range of 2–120 nm.

5.2.4 Uranium Nanoparticles

Cell-free extracts of *Micrococcus lactilyticus* have been reported to reduce uranium (VI) to uranium (IV) (Woolfolk and Whiteley 1962). Also, *Alteromonas putrefaciens* grown in the presence of hydrogen as electron donor and U (VI) as electron acceptor reduced U(VI) to U(IV) (Myers and Nealson 1988; Lovley et al. 1989).

Lovley et al. (1991) demonstrated that *G. metallireducens* GS-15, grown anaerobically in the presence of acetate and U(VI) as electron donor and electron acceptor, respectively, reduced soluble U(VI) to insoluble U(IV), oxidizing acetate to CO_2. The Gram-positive sulfate-reducing bacterium *Desulfosporosinus sp.*, isolated from sediments, has been found to reduce U(VI) to U(IV), which is precipitated to yield uraninite (UO_2) crystals of 1.5–2.5 nm size range, coated on the cell surface (Suzuki et al. 2002). Marshall et al. (2006) found that c-type cytochrome (MtrC) on the outer membrane of dissimilatory metal-reducing bacterium *S. oneidensis* MR-1 is involved in the reduction of U(VI), predominantly with extracellular polymeric substance as UO_2-EPS, both in cell suspension and periplasm.

5.2.5 *Cadmium Nanoparticles*

Cadmium is primarily used in the synthesis of particles called quantum dots (QDs), which are semiconductor metalloid-crystal structures of approximately 2–100 nm and containing about 200–10,000 atoms (Smith et al. 2008; Juzenas et al. 2008). Due to their small size, QDs have unique optical and electronic properties that impart the nanoparticles with a bright, highly stable, "size-tunable" fluorescence. The large surface area due to their small size also makes QDs easily functionalized with ligands for site-directed activity. Thus, QDs have potential applications in biological imaging at the cellular level, cancer detection, radio- and chemosensitizing, and targeted drug delivery (Juzenas et al. 2008; Alivisatos 2004; Smith et al. 2008; Hardman 2006). The active center of the QD demonstrated as the core consists of atoms from groups II to VI with CdSe and CdTe, most commonly used for biological applications (Smith et al. 2008). The significant characteristic of QDs is their size-tunable fluorescence. They are significantly brighter than organic fluorophores and far more stable. Since the fluorescence is size-dependent, a single light source can be used for excitation and emission, which is tuned via particle size to various wavelengths spanning the UV, visible, and near and mid-infrared regions of the electromagnetic spectrum. Unlike organic fluorophores, QDs are also much larger, permitting easy addition of targeting groups to the surface of the nanoparticle. CdSe and CdTe are important for optical, bioananalytic, and bioimaging applications, with CdSe fluorescence spanning the visible light region of the spectrum and CdTe utilizing the infrared regions. Since the QDs are hydrophobic, their functionalization with secondary coatings or "capping" materials such as mercaptopropionic acid and polyethylene glycol (PEG) is required to improve solubility and maintain them in a nonaggregated state. These coatings can be further conjugated with targeting molecules such as receptor ligands or antibodies, which guide the QD to a specific tissue or organ (Medintz et al. 2005; Smith et al. 2008). Thus, QDs have the potential to dramatically improve medical therapy with respect to cancer detection and treatment.

Among early reports of intracellular semiconductor nanoparticle synthesis, *E. coli* has been found to accumulate nanocrystals composed of wurtzite crystal in

the size range of 2–5 nm, with spherical and elliptical shapes when incubated with cadmium chloride and sodium sulfide. The production of nanocrystals is reported to be 20-fold higher when the *E. coli* cells are grown to the stationary phase as compared to late logarithmic phase. It has also been found that spherical aggregates of 2–5 nm diameter sphalerite (ZnS) particles are formed within natural biofilms dominated by aerotolerant sulfate-reducing bacteria of the family *Desulfobacteriaceae* (Labrenz et al. 2000). Among semiconductor nanocrystals, CdS synthesized by *C. thermoaceticum*, *Klebsiella pneumoniae* (Smith et al. 1998), and *E. coli* (Sweeney et al. 2004) showed spherical and elliptical shapes in the size range of 2–200 nm. Sharma et al. (2000) isolated a highly cadmium-resistant *Klebsiella planticola* strain Cd-1 from reducing salt marsh sediments. The strain could grow in up to 15 mM $CdCl_2$ under a wide range of NaCl concentrations and at pH values ranging from acidic to neutral. In growth media amended with thiosulfate, the strain precipitates significant amounts of cadmium sulfide (CdS), as confirmed by X-ray absorption spectroscopy. *Klebsiella aerogenes* synthesized CdS crystallites of spherical shape, bound to the cell wall as electron-dense particles in the size range of 20–200 nm, upon exposure to Cd^{2+} in the growth medium. Energy dispersive X-ray analysis has established that cadmium and sulfur occur in a 1:1 ratio (Holmes et al. 1995). Bai et al. (2009) showed that *Rhodopseudomonas palustris*, a purple nonsulfur, photosynthetic bacterium, produced CdS nanocrystals extracellularly at room temperature. TEM and electron diffraction analyses confirmed the spherical distribution of fcc structured nanoparticles of 8.01 ± 0.25 nm size. Cysteine desulfhydrase (C-S lyase) activity has been reported to be responsible for the formation of CdS nanocrystals. The bacterial cellulose isolated from the strain *Gluconoacetobacter xylinus* has also been used in the synthesis of 30-nm CdS nanoparticles (Li et al. 2009).

5.2.6 Selenium Nanoparticles

Considering selenium oxyanions as the electron acceptor, bacteria such as *Sulfurospirillum barnesii*, *B. selenitireducens*, and *Selenihalanaerobacter shriftii* form uniform and stable crystalline extracellular nanoparticles of Se nanoparticles measuring ~300 nm. The spectral properties of nanoparticles differ significantly from that of amorphous Se^0 formed by the chemical oxidation of H_2Se and the vitreous (black) Se^0 formed chemically by reduction of selenite with ascorbate. Oremland et al. (2004) reported the structural and spectral features of selenium nanospheres produced by Se-respiring bacteria. *Stenotrophomonas maltophilia* SELTE02, a strain isolated from rhizospheric soil of selenium hyperaccumulator legume *Astragalus bisulcatus*, showed promising transformation of selenite (SeO_3^{2-}) to elemental selenium (Se^0) and accumulation of selenium granules in either the cell cytoplasm or extracellular space (Gregorio et al. 2005). Also, the facultative anaerobic bacterium, *E. cloacea* SLD1a-1 (Losi and Frankenberger 1997), purple nonsulfur bacterium *Rhodospirullum rubrum* in oxic and anoxic conditions, and *Desulfovibrio*

desulfuricans (Tomei et al. 1995) are reported to bioreduce selenite to selenium both inside and outside the cell. *E. coli* has also been found to deposit elemental selenium both in the periplasmic space and cytoplasm (Gerrard et al. 1974; Silverberg et al. 1976). *P. stutzeri* is also known to aerobically reduce selenite to elemental selenium (Lortie et al. 1992). Recently, Yadav et al. (2008) have showed that *P. aeruginosa* SNT1, isolated from rhizospheric seleniferous soil, biosynthesized nanostructured selenium by biotransforming selenium oxyanions both intracellularly and extracellularly to spherical amorphous allotrophic elemental red selenium. Selenium has photo-optical and semiconducting properties and, therefore, has applications in photocopiers and microelectronic circuit devices.

5.2.7 Titanium, Platinum, and Palladium Nanoparticles

The extracellular culture filtrate of *Lactobacillus* sp. has been shown to produce titanium nanoparticles at room temperature in the form of spherical aggregates ranging in size from 40 to 60 nm (Prasad et al. 2007). Titanium dioxide (TiO_2) nanoparticles are lighter in weight and resistant to corrosion and, therefore, have widespread applications in automobiles, missiles, airplanes, submarines, cathode ray tubes, and in desalting plants, besides a promising role in gene delivery and cancer chemotherapy. TiO_2 nanoparticles also exhibit photocatalytic activities, and therefore, are recommended for use as antibacterial agents, UV protecting agents, water and air purifiers, and in gas sensors and high-efficiency solar cells. Its photoactivity is strongly related to its structure, microstructure, and powder purification. The three known crystalline structures for TiO_2 include the anatase (tetragonal, a = 0.3785 nm, c = 0.9514 nm, band gap = 3.2 eV, which is equivalent to a wavelength of 388 nm), rutile (tetragonal, a = 0.4593 nm, c = 0.2959 nm, band gap = 3.02 eV), and brookite (orthorhombic, a = 0.9182 nm, b = 0.5456 nm, c = 0.5143 nm, band gap = 2.96 eV). The anatase form of TiO_2 has more photocatalytic activity than rutile. The rutile is thermodynamically more stable than anatase and brookite.

The Gram-negative Cyanobacterium *P. boryanum* UTEX 485 has been reported to produce extracellular Pt (II)-organics and metallic platinum nanoparticles with spherical, bead-like chains, and dendritic morphologies in the particle size range of 30–300 nm. Stationary phase culture of metal ion-reducing bacterium *Shewanella algae* in aqueous solution of H_2PtC_{l6}, under anaerobic conditions at room temperature and neutral pH, has been shown to reduce $PtCl_6^{2-}$ ions within 60 min to metallic platinum in the presence of lactate as electron donor. Platinum nanoparticles of ~5 nm size have been observed deposited in the periplasmic space between inner and outer membranes of the bacterial cell (Konishi et al. 2007). Also, the sulfate-reducing bacterium *D. desulfuricans* NCIMB 8307 anaerobically bioreduced and biocrystallized palladium (2+) ions to palladium nanoparticles on the cell surface in the presence of formate as an exogenous electron donor within minutes at neutral pH (Yong et al. 2002). De Windt et al. (2005) have demonstrated

that an iron-reducing bacterium, *S. oneidensis* MR-1, reduced Pd(II) to Pd(0) nanoparticles in the presence of lactate as electron donor on the cell wall and within the periplasmic space. This cell-associated nano-bioPd has an application as a catalyst in the dechlorination of polychlorinated biphenyls.

5.3 Nanoparticle Biosynthesis by Actinomycetes

Actinomycetes are generally considered as the primary source for the synthesis of secondary metabolites like antibiotics. However, screening of actinomycetes for their innate potential for nanoparticle synthesis is an area open for further exploration. An extremophilic actinomycete *Thermomonospora* sp. has been reported to synthesize extracellular monodispersed, spherical gold nanoparticles of average size 8 nm (Ahmad et al. 2003a). Fourier transform infrared spectroscopic (FTIR) analysis confirmed the presence of amide (I) and (II) bands of protein as capping and stabilizing agent on the surface of nanoparticles. Furthermore, an alkalotolerant actinomycete *Rhodococcus* sp. accumulated intracellularly gold nanoparticles of 5–15 nm. The available reductases on the cell wall reduced Au^{3+} and accumulated Au^{0} on the cell wall and cytoplasmic membrane.

5.4 Nanoparticle Biosynthesis by Cyanobacteria

The cyanobacterium *Plectonema boryanum* UTEX 485 has been found to produce silver nanoparticles. Also, this filamentous cyanobacterium upon incubation with aqueous $Au(S_2O_3)^2$ and $AuCl_4$ solutions produced cubic gold nanoparticles and octahedral gold platelets, respectively (Lengke et al. 2006a, b). The mechanism of gold bioaccumulation by cyanobacteria from gold (III)-chloride solution suggested that its interaction with cyanobacteria promotes the precipitation of nanoparticles of amorphous gold (I)-sulfide at the cell wall, and finally deposited metallic gold in the form of octahedral (III) platelets (10 nm to 6 μm) near cell surfaces and in solution (Lengke et al. 2006a, b). Some common *Anabaena*, *Calothrix*, and *Leptolyngbya* cyanobacteria have also been found to produce intracellular Au, Ag, Pd, and Pt nanoparticles, which naturally released in the culture medium and stabilized by algal polysaccharides for their easy recovery. Indeed, the size of the recovered particles and yield depend on the cyanobacteria genus (Brayner et al. 2007).

5.5 Nanoparticle Biosynthesis by Yeast

The yeast *Candida glubrata* has been used for the intracellular production of monodispersed spherical and peptide-bound CdS quantum dots measuring 2 nm, by forming a metal–thiolate complex with phytochelatins (Dameron et al. 1989).

Schizosaccharomyces pombe also produced wurtzite-typed hexagonal lattice structured CdS nanoparticles in mid-log phase in the size range of 1–1.5 nm (Kowshik et al. 2002). The synthesis of fcc structured PbS nanocrystallites exhibiting quantum semiconductor properties by yeast *Torulopsis sp.* has been reported (Kowshik et al. 2002). The quantum dots are intracellularly produced in the vacuoles with a dimension of 2–5 nm spherical shape. These nanoparticles are used to fabricate diode heterojunction with poly (*p*-phenylenevinylene). In addition, Baker's yeast, *Saccharomyces cerevisiae*, has been reported to biosorb and reduce Au^{3+} to elemental gold in the peptidoglycan layer of the cell wall by the aldehyde group present in reducing sugars (Lin et al. 2005). Similarly, another yeast, *Pichia jadinii* (*Candida utilis*), intracellularly produced spherical, triangular, and hexagonal gold nanoparticles of 100 nm size within 24 h (Gericke and Pinches 2006). Another tropical marine yeast, *Yarrowia lipolytica* NCIM 3589, produced hexagonal and triangular gold crystals of average size ~15 nm, nucleated on the cell surfaces by reduction of gold ions at pH 2.0. Further, *S. cerevisiae* has been found to produce spherical antimony oxide (Sb_2O_3) nanoparticles in the size range of 2–10 nm at room temperature, exhibiting semiconductor properties. The plausible mechanism could be the radial tautomerization of membrane-bound quinines or by membrane bound/cytosolic pH-dependent oxidoreductases (Jha et al. 2009). Extracellular production of hexagonal silver nanoparticles 2–5 nm in size has also been reported in the silver-tolerant yeast strain MKY3 in the exponential growth phase (Kowshik et al. 2003).

5.6 Nanoparticle Biosynthesis by Fungi

Biosynthesis of metal nanoparticles using fungi such as *F. oxysporum* (Senapati et al. 2004; Bansal et al. 2004, 2005; Kumar et al. 2007), *Colletotrichum* sp. (Shankar et al. 2003), *Trichothecium* sp., *Trichoderma asperellum*, *T. viride*, (Ahmad et al. 2005; Mukherjee et al. 2008; Fayaz et al. 2010), *Phaenerochaete chrysosporium* (Vigneshwaran et al. 2006), *Fusarium solani USM3799* (Ingle et al. 2009), *Fusarium semitectum* (Basavaraja et al. 2008), *Aspergillus fumigatus* (Bhainsa and D'Souza 2006), *Coriolus versicolor* (Sanghi and Verma 2009), *Aspergillus niger* (Gade et al. 2008), *Phoma glomerata* (Birla et al. 2009), *Penicillium brevicompactum* (Shaligram et al. 2009), *Cladosporium cladosporioides* (Balaji et al. 2009), *Penicillium fellutanum* (Kathiresan et al. 2009), and *Volvariella volvacea* (Philip 2009) has been extensively studied. Indeed, fungi are regarded as more advantageous for nanoparticle biosynthesis as compared to other microorganisms because (1) fungal mycelial mesh can withstand flow pressure, agitation, and other conditions in bioreactors compared to bacteria, (2) they are fastidious to grow and easy to handle, and (3) they produce more extracellular secretions of reductive proteins and can easily undergo downstream processing. Moreover, the nanoparticles precipitated outside the cell can be directly used in various applications. The size limit of nanoparticles could be related to the fact that the particles nucleate within the organism. Such nanoparticles could be smaller

compared to extracellularly produced nanoparticles. Mukherjee et al. (2001b) demonstrated the biological synthesis of 20-nm gold nanoparticles using *Verticillium* sp. (AAT-TS-4). TEM analysis of ultrathin sections of fungal mycelia showed mostly spherical forms and few triangles and hexagonal nanoparticles on cell walls and quasi-hexagonal morphology on cytoplasmic membranes. In addition, *Verticillium luteoalbum* has been reported to produce spherical 10-nm gold nanoparticles within 24 h at pH 3.0. However, at pH 5.0, spheres and rods were formed along with triangular and hexagonal morphologies (Gericke and Pinches 2006). *Trichothecium* sp. has also been found to accumulate gold nanoparticles intracellularly (Dastjerdi et al. 2009).

Furthermore, *Verticillium* sp. Biomass, on exposure to aqueous silver nitrate solution, resulted in accumulation of silver nanoparticles beneath the fungal cell surface (Mukherjee et al. 2001a; Senapati et al. 2004). Phoma PT35 and Phoma sp.3.2883 have been shown to selectively accumulate silver nanoparticles (Pighi et al. 1989; Chen et al. 2003). Vigneshwaran et al. (2007) reported that *Aspergillus flavus* accumulated silver nanoparticles 8.9 nm in size on the surface of its cell wall when incubated with silver nitrate solution for 72 h. Since fungi are known to secrete much higher amounts of proteins compared to bacteria, it could be one of the contributory factors for significantly higher productivity of nanoparticles in this biosynthetic approach. In order to elucidate the mechanism of nanoparticle formation, species-specific NADH-dependent reductase, released by *F. oxysporum*, has been used to catalyze the reduction of $AuCl_4$ ions to gold nanoparticles. The trapping of $AuCl_4$ ions on the surface of fungal cells could occur by electrostatic interactions with positively charged lysine residues present in the mycelia cell wall. The gold ions could be reduced by enzymes within the cell wall, leading to aggregation of metal atoms; however, the exact mechanism of formation of the gold nanoparticles is still unknown. It has been suggested that extracellularly produced nanoparticles are stabilized by proteins and other reducing agents secreted by the fungus. Experimental data suggest the association of some high-molecular-weight proteins including the NADH-dependent reductase released by fungal biomass in nanoparticle synthesis and stabilization. Fluorescence emission spectra reveal that the native form of these proteins present in solution as well as bound to the surfaces of nanoparticles remains unaltered and the reduction of metal ions did not significantly influence protein tertiary structure (Macdonald and Smith 1996; Kumar and McLendon 1997).

Proteins isolated from fungal cultures have been successfully used to demonstrate nanoparticle production. For instance, nanocrystalline zirconia has been produced at room temperature by cationic proteins, similar in nature to silicatein, secreted by *F. oxysporum* and was capable of extracellularly hydrolyzing aqueous ZrF_6 ions (Bansal et al. 2004).

Growth conditions play an important role during biosynthesis of nanoparticles. *Trichothecium* sp. biomass under stationary conditions produced extracellular nanoparticles when incubated with gold ions. However, under agitation, the fungus produces intracellular gold nanoparticles. The plausible reason for this could be the release of enzymes and proteins responsible for nanoparticle synthesis in the

medium under stationary conditions and no release under shaking conditions (Ahmad et al. 2005). Bharde et al. (2006) reported the synthesis of magnetic nanoparticles by using *F. oxysporum* and *Verticillium* sp. at room temperature. Both fungi secreted the proteins capable of hydrolyzing iron precursors to form iron oxides extracellularly (Gericke and Pinches 2006). Bhainsa and D'Souza (2006) have reported the production of monodispersed silver nanoparticles within 10 min using *A. fumigatus*. Also, Bansal et al. (2006) demonstrated the production of tetragonal barium titanate ($BaTiO_3$) nanoparticles of <10 nm dimension using *F. oxysporum* under ambient conditions. The ferroelectric properties of these nanoparticles have tremendous potential for revolutionizing the electronics industries with their applications in preparing ultrasmall capacitors and ultrahigh density nonvolatile ferromagnetic memories. Furthermore, Bansal et al. (2005) and Kumar et al. (2007) have reported the synthesis of highly luminescent CdSe quantum dots, and silica and titania nanoparticles using the fungus *F. oxysporum.*

5.7 Scope and Applications of Nanoparticles

Production of inorganic and metal-based nanomaterials has stimulated the development of a new field linking many disciplines of sciences for the quest for different types of nanoparticles with unique properties. Designing and developing novel and affordable techniques for scale-up production of nanomaterials have not only provided an interesting area of study but will also address the expanding human requirements including health safety and environmental issues. In industry, the application of nanomaterials is increasingly adopted, and they will soon replace the harmful or toxic chemicals conventionally used as antimicrobial agents (Mucha et al. 2002). Application of nanoparticles and their nanocomposites offers a sound and relatively safer alternative (Chen et al. 2006; Dimitrov 2006) and, therefore, open up new opportunities for development of antimicrobials. Since ancient times, silver has been most extensively studied and used to fight against infection and prevent spoilage (Rai et al. 2009). It is a safer antimicrobial agent in comparison to certain organic antimicrobial agents (Dastjerdi et al. 2010). Silver has been described as being oligodynamic because of its ability to exert a bactericidal effect on products containing silver, principally due to its antimicrobial activities and low toxicity to human cells (Dastjerdi et al. 2009). Its therapeutic property has been proven against a broad range of microorganisms (Jeong et al. 2005; Lok 2006). Lately, Musarrat et al. (2010) demonstrated the broad-spectrum antimicrobial activity of biosynthesized AgNPs against several human and plant pathogenic bacteria and fungi such as *Shigella dysenteriae* type I, *Staphylococcus aureus*, *Citrobacter* spp., *E. coli*, *P. aeruginosa*, *B. subtilis*, *Candida albicans,* and *F. oxysporum*.

Similarly, ZnO nanoparticles and nanorods have remarkable applications in solar cells, sensors, displays, gas sensors, piezoelectric devices, electroacoustic transducers, photodiodes and UV light emitting devices, sunscreens, gas sensors, UV absorbers, antireflection coatings, photocatalysis, and chemical catalysts

(Pan et al. 2001; Xu and Xie 2003). Gold nanoparticles are also known for their potent antibacterial activity against acne or scurf and have commercial applications in soap and cosmetic industries. They can remove waste materials from skin and control sebum (Park et al. 2006; Zhang et al. 2008). Zhang et al. (2008) have reported Au nanoparticle-mediated growth inhibition of different Gram-positive and Gram-negative bacteria and fungi. Park et al. (2006) loaded gold nanoparticles inside the liposomes, which could be used as a controlled release delivery system.

Nanoparticles have enormous applications in biology and medicine. In a dynamic range of size <100 nm, they could be used as probes attached to peptides, antibodies, or nucleic acids for detection and quantification of molecular reactions in vivo (Niemeyer 2001). The potential for coating nanoparticles with antibodies, collagen, and other substances makes them biocompatible for detection and medical diagnosis. Bruchez et al. (1998) showed that nanoparticle-based fluorescent labeling is superior to the use of conventional fluorophores. Wu et al. (2003) observed that quantum-dot-based immunofluorescent labeling of the cancer marker Her2 is more efficient than normal fluorophores in labeling different target cell-surface receptors, nuclear antigens, the cytoskeleton, and other intracellular organelles. They also demonstrated that bioconjugated colloidal quantum dots were valuable in cell labeling, cell tracking, DNA detection, and in vivo imaging. Zhang et al. (2002) showed that surface modification of superparamagnetite nanoparticles with ethylene glycol and folic acid is effective in facilitating phagocytosis by cancer cells for potential cancer therapy and diagnosis. O'Neal et al. (2004) observed in mice that selective photothermal ablation of tumors using near infrared-absorbing polyethylene-coated gold nanoshells of 130 nm size inhibited tumor growth and increased survival of animals for up to 90 days compared with controls. Moreover, the antibody-coated magnetic iron nanoparticles reported by Perkel (2004) have been proven very effective to heat and virtually burn tumors. Gopalan et al. (2004) reported nanoparticle-based gene therapy using a novel tumor suppressor gene, FUSI, to be effective in systemic gene treatment of lung cancer. Dufes et al. (2005) reported gene therapy by intravenous administration of nanoparticle-based vector systems using tumor necrosis factor (TNF)-α expression plasmid and found increased transgene expression and long-term survival of rats with no toxicity. In vitro studies with breast cancer cells have shown the efficacy of nanoparticle-mediated gene delivery of the wild-type p53 gene. Cancer cells, upon nanoparticle-based gene delivery, exhibited an increased and sustained antiproliferative activity. Kaul and Amiji (2005) observed that PEG-modified gelatin nanoparticles used for tumor-targeted gene delivery have been highly effective, biocompatible, biodegradable, and long-circulating for systemic delivery to solid tumors.

Pathogen detection is another widely explored area in BioMEMS research. Culture and colony counting methods and PCR have been the two conventional and most selective/reliable methods in molecular biology laboratories, although they take hours to days to provide conformity. The emphasis of detection technologies has been moved to BioMEMS/sensor technology because this provides equally reliable results in a fraction of the time employed for conventional methods.

5.8 Conclusions

During the past several years, various methods based on chemical reduction, thermal treatment, irradiation and laser ablation, etc. have been used for synthesis of metal nanoparticles. Most of these methods rely heavily on the use of organic solvents and toxic reducing agents like sodium borohydride and N, N-dimethylformamide, which may pose severe environmental problems and biological risks. Therefore, biological and biomimetic approaches for green synthesis of nanomaterials are now highly appealing, utilizing the potential of bacteria, fungi, and even plants for nanoparticle synthesis as eco-friendly nanofactories. The cell mass and leached components from microorganisms have reportedly been utilized for the reduction of metal ions to nanoparticles, through enzymes such as oxidoreductases and a shuttle quinone extracellular process. Filamentous fungi possess some distinctive advantages over bacteria due to ease of handling, mass cultivation, high metal tolerance, wall-binding capacity, and intracellular metal uptake capabilities. Nanoparticles of noble metals like gold, platinum, palladium, and silver, etc. have attracted scientific attention in recent years due to their unique chemical and physical attributes that differ from the respective bulk substance. The extremely small size and large surface area relative to their volume make them useful for many applications viz. nonlinear optics, spectrally selective coatings for solar energy absorption, optical receptors, catalysis in chemical reactions, biolabelling, and as antibacterials. Thus, the use of biologically compatible materials for nanoparticle synthesis and stabilization could play a crucial role in medical diagnosis and therapeutics including the detection of genetic disorders by color-coded fluorescent labeling of cells using semiconductor quantum dots and cell transfection for gene therapy and drug delivery.

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Chapter 6
Bacterial Quorum Sensing and Its Interference: Methods and Significance

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Abstract Bacteria use the language of low-molecular-weight ligands to assess their population densities in a process called quorum sensing (QS). Different types of quorum sensing pathways are present in Gram-negative and Gram-positive bacteria. Signal molecules most commonly used in Gram-negative bacteria are acyl homoserine lactones. In recent years, a substantial amount of literature and data have been available on bacterial QS. Recently, interest in modulation of quorum sensing with different approaches has increased among scientific communities. In this chapter, we provide an updated overview on bacterial QS, assays and methods for detecting signal molecules, and various approaches to inhibit AHL-based quorum sensing. Significance of QS interference by prokaryotic and eukaryotic organisms in relation to plant health and the environment is discussed here.

6.1 Introduction

The first evidence of cooperative behavior among bacteria was described almost 50 years ago by Tomasz (1965). Nealson et al. (1970) studied the biology of light-producing organelles, i.e., via the bacterium *Vibrio fischeri* in deep-sea fish through a cell-density-dependent reaction. Subsequently, this population-dependent phenomenon was termed "quorum-sensing" by Fuqua et al. (1994), reflecting the minimum threshold level of individual cell mass required to initiate a concerted

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_6,

population response. Such a system is coordinated by the intracellular production and export of a low-molecular-mass signaling molecule, the extracellular concentration of which increases with the population density of the producing organism. The signal molecules involved in such communication are termed "autoinducers," owing to their origin inside the bacterial cell and regulating their own expression. The signaling molecule can be sensed and reimported into these cells, thus allowing the entire population to respond to changing environment/requirements once a critical concentration (corresponding to a particular cell density) or "quorum," i.e., the minimal number of bacteria amassed within a volume to make the "decision" to switch on gene expression of QS-controlled genes, is attained.

In Gram-negative bacteria, the best-studied QS systems use LuxI-type enzymes, which produce *N*-acyl homoserine lactones (AHLs) as signal molecules, which bind and activate members of the LuxR transcriptional activator protein family (Juhas et al. 2005; Duan and Surette 2007).The autoinducer *N*-(3-oxohexanoyl)-*N*-homoserine lactone (3-oxo-C6-HSL) was identified as one of the quorum signaling molecules for *V. fischeri*. This molecule is the product of the *LuxI* autoinducer synthase, which catalyzes the reaction between *S*-adenosylmethionine and acylated-acyl carrier proteins to produce 3-oxo-C6-HSL. The *luxI* gene resides in the rightward portion of the bidirectional *lux* operon (*luxICDABEG*) containing both *luxI* and the genes encoding the proteins involved in bioluminescence. The *luxR* gene, which encodes the 3-oxo-C6-HSL-dependent response regulator, is encoded in the left operon (Egland and Greenberg 1999).

6.2 Quorum Sensing Pathways in Bacteria

A remarkable array of signaling molecules function as local sensors to communicate population densities in Gram-negative and Gram-positive bacteria. These molecular signals and their receptors are broadly grouped in up to four QS systems.

6.2.1 Autoinducer Type 1 Signaling System

The autoinducer type 1 system is widely used in multiple genera of Gram-negative bacteria and is highly homologous to the original *luxR/luxI* autoinducer type 1 system in *Vibrio* spp., first defined in *V. fischeri* (Beutler et al. 2006). A highly soluble and freely diffusible sensor molecule uses a series of *N*-acyl homoserine lactone (AHL) molecules for signaling. AHL molecules vary in the *N*-acyl chain length (from 4 to 18 carbons), degree of saturation, and number of oxygen substitutions. The L-isomeric form of the homoserine lactone ring is common to all AHLs. The *luxI* gene or its homologues encode the sequences that mediate the formation of the AHL signaling molecule.

The receptor for AHL is mediated by the *luxR* gene in *V. fischeri* or related genes in other bacterial species. The translated product of *luxR* is the LuxR receptor molecule, which together with its AHL partner functions as a coactivator complex at the promoter sites for QS responsive operons in the bacterial genome. In *V. fischeri*, the end result is bioluminescence; for certain bacterial pathogens, the end product is the activation of many virulence factors.

Some of the bacterial genera use the same AHL molecule, indicating that some level of interspecies cross talk exists (Williams 2007). Other bacteria including *E. coli* do not synthesize AHLs but express a LuxR biosensor homologue (SdiA). It is speculated that this sensing system allows *E. coli* to "listen" to communication signals from other Gram-negative bacteria and exploit this information to its own advantage (Ahmer 2004).

6.2.2 Autoinducer Type 2 Signaling System

A second QS pathway was initially discovered in the *V. harveyi* bioluminescence system and is mediated by the *luxS* gene locus and related homologues (Miller and Bassler 2001; Bassler 2002). Elements of the autoinducer type 2 (AI-2) system are detectable in almost one half of all sequenced bacterial genomes, and this system is now recognized as the most ubiquitous signaling system employed by both Gram-negative and Gram-positive bacteria (Wen and Burne 2004; Hermann 2007). The AI-2 pathway uses a more complex, two component, receptor kinase network to accomplish efficient signaling among bacteria. Structurally, the AI-2 signal in *Vibrio* spp. is composed of rather complex, multiple-ringed, cyclical furanosyl molecules containing the highly unusual presence of a boron atom (Bassler 2002). The receptor for the AI-2 apparatus is also complex, with a series of gene products that function as the receptor kinase signal transcription complex. In *Vibrio* species, the receptor is a membrane-bound, two-domain, sensor kinase and response regulator (LuxQ). In enteric bacteria, a soluble receptor binds to the AI-2 signal molecule in the periplasmic space and then transports the AI-2 molecule across the membrane via a specific ABC-type transporter system (Kendall and Sperandio 2007). The internalized AI-2 molecule is phosphorylated and then complexed with an intracellular receptor that acts as the transcriptional activator.

Multiple variations of this AI-2 system are found in bacteria (Shiner et al. 2005). The AI-2 signaling molecule in *Salmonella* spp. is a furan molecule that lacks boron. Many bacteria apparently do not express the *luxS* gene but express the AI-2 receptor complex (Kendall et al. 2007). Such an arrangement has been proposed to allow some bacterial strains to sense and use AI-2 signals generated by other bacteria to regulate their own coordinated transcriptional responses (Li et al. 2008). The precise role of AI-2 signaling in bacterial pathogenesis is not clear, as much of the transcriptional activity of AI-2 systems is directed toward regulation of metabolic pathways (Zhao et al. 2010).

6.2.3 Autoinducer Type 3 System

This newly discovered QS system is perhaps the most complicated of all signaling pathways thus far discovered. The autoinducer type 3 (AI-3) system shares many characteristics of the AI-2 system, as it uses a two-component, receptor kinase intracellular signaling complex to activate genes of the virulome; however, in contrast to AI-2, the AI-3 system can use the human stress hormones epinephrine or norepinephrine to signal the system (Walters and Sperandio 2006; Kendall et al. 2007).

The periplasmic receptor for the AI-3 system has recently been characterized and is known as the QseBC complex (Clarke et al. 2006). QseC is the sensor kinase, and QseB is the phosphorylated response regulator that alters transcription of virulence genes. The AI-3 system is essential in the pathogenesis of enterohemorrhagic *E. coli* infections and shigellosis (Kendall et al. 2007). Components of the AI-3 signaling network have been detected in enteropathogenic *E. coli* strains, commensal *E. coli* strains, and a number of other Gram-negative, enteric organisms, but thus far not in Gram-positive bacteria.

6.2.4 Short Peptide Signaling (AIP) System in Gram-Positive Bacteria

A number of Gram-positive bacteria are also known to employ quorum-sensing systems. The nature of the signal molecules used in these systems differs from those of Gram-negative organisms (de Kievit and Iglewski 2000). Many cell–cell signaling systems in Gram-positive bacteria use modified peptides as signals to regulate functions such as virulence (agr system in staphylococci – Ji et al. 1995) and fsr system in enterococci – Haas et al. 2002), competence (com system in bacilli – Hamoen et al. (2003) and (pneumococci – Tomasz, 1965; Havarstein et al. 1995), and bacteriocin production (pin and ssp systems in lactic acid bacteria). Most autoinducing peptide (AIP) signals are generated by cleavage from larger precursor peptides, and subsequent modifications including substitution with isoprenyl groups and formation of lactone and thiolactone rings and lanthionines (Ansaldi et al. 2002). Signal release from the cell requires dedicated oligopeptide exporters, whereas signal perception is mediated by sensor histidine kinases located in the cytoplasmic membrane. Many Gram-positive bacteria communicate with multiple peptides in combination with other types of quorum-sensing signals. In some cases, the signaling peptide can be recognized not only by its cognate species but also by different strains of the same or related species (Thoendel and Horswill 2010).

6.3 QS Signal Molecules Diversity

Most QS signals are either small (1,000 Da) organic molecules or peptides with 5–20 amino acids (Chhabra et al. 2005; Williams 2007). Gram-negative bacteria, for example, employ N-acyl homoserine lactones (AHLs), 2-alkyl-4-quinolones

(AQs), long-chain fatty acids, and fatty acid methyl esters, as well as autoinducer-2 (AI-2), a collective term for a group of interconvertible furanones derived from dihydroxypentanedione (DPD). AI-2 is also produced by some Gram-positive bacteria, although generally these organisms prefer linear, modified, or cyclic peptides such as the autoinducing peptides (AIPs) made by the staphylococci. The streptomycetes, however, synthesize c-butyrolactones such as A-factor, which are structurally related to the AHLs, as both compound classes belong to the butanolides. In general, however, Gram-positive bacteria engage post-translationally modified peptides as quorum-sensing signal molecules referred as autoinducing peptides (AIPs), which range from 5 to 34 amino acids in length and typically contain unusual chemical architectures. Based on their structural uniqueness, three different families of AIPs have been characterized: (a) the oligopeptide lantibiotics, typified by the lactococcal nisin, which are characterized by the presence of lanthionine-mediated thioether macrocyclic features and dehydroamino acid residues (Quadri 2002), (b) the 16-membered thiolactone peptides, exemplified by the staphylococcal AIP-1 (Chan et al. 2004), and (c) the isoprenylated tryptophan peptides in which ComX and its variants from *Bacillus subtilis* and other *Bacillus* species are currently the only known members (Ansaldi et al. 2002; Okada et al. 2005). QS signal molecules can also be further subdivided according to whether they interact with receptors at the cell surface (e.g., the staphylococcal AIPs) or are internalized (e.g., the AHLs, AQs, the Phr peptides of *Bacillus subtilis*, and the mating pheromones of *Enterococcus faecalis* (Williams 2007). However, here, we primarily consider quorum-sensing signal molecules and their inhibition in Gram-negative bacteria.

6.3.1 Gram-Negative Bacteria

AHL-mediated quorum sensing is employed by diverse Gram-negative proteobacteria belonging to α, β, and γ subdivisions (Chhabra et al. 2005). Most AHL-producers synthesize multiple AHLs that are characterized by a homoserine lactone (HSL) unsubstituted in the β- and γ-positions, which is *N*-acylated with a fatty acyl group at the α-position. The acyl chain varies in length, saturation level, and oxidation state. In most cases, the chain has an even number of carbons (C4–C18), although AHLs with C5 and C7 acyl chains have been identified (Lithgow et al. 2000; Horng et al. 2002). Examples of different AHLs produced by Gram-negative bacteria are shown in Table 6.1. They belong either to the *N*-acyl or *N*-(3-oxoacyl) or *N*-(3-hydroxyacyl) classes of compounds. AHLs with C14 and C18 acyl chains have also been described, which also contain one or two double bonds (Wagner-Dobler et al. 2005).

AHL-mediated signaling appears to require at least a C4 acyl side chain since *N*-butanoylhomoserine lactone (C4-HSL) and *N*-hydroxybutanoylhomoserine lactone (3-hydroxy-C4-HSL) are the shortest AHLs found naturally (Winson et al. 1995). This is probably because the HSL ring is highly susceptible to pH-dependent ring opening, a susceptibility which decreases as the acyl side chain is lengthened. Consequently, HSL and *N*-propionylhomoserine lactone (C3-HSL) are rapidly hydrolyzed at pH values well below 7.0. The HSL ring, for example, is typically

Table 6.1 Some examples of AHL-dependent QS systems and the phenotypes controlled [adapted from Williams (2007)]

Organism	AHLs	Phenotype
Aeromonas hydrophila	C_4-HSL, C_6-HSL	Biofilm, exoprotease, virulence
Aeromonas salmonicida	C_4-HSL, C_6-HSL	Exoprotease
Agrobacterium tumefaciens	3-OXO-C_8-HSL	Plasmid conjugation
Burkholderia cenocepacia	C_6-HSL, C_8-HSL	Exoenzymes, biofilm formation, swarming motility, siderophore, virulence
Chromobacterium violaceum	C_6-HSL	Exoenzyme, cyanide, pigment
Erwinia carotovora	3-OXO-C_6-HSL	Carbapenem, exoenzyme, virulence
Nitrosomonas europea	3-OXO-C_6-HSL	Emergence from lag phase
Pseudomonas aeruginosa	C_4-HSL, C_6-HSL, 3-OXO-C_{12}-HSL	Exoenzymes, exotoxins, protein secretion, biofilms, swarming motility, virulence
Pseudomonas aureofaciens	C_6-HSL	Phenazines, protease, colony morphology, aggregation, root colonization
Pseudomonas putida	3-OXO-C_{10}-HSL, 3-OXO-C_{12}-HSL	Biofilm development
Pseudomonas flourescens	3-OXO-C_{10}-HSL	Mupirocin
Rhizobium leguminosarum bv. Viciae	C_{14}-HSL, C_6-HSL, C_7-HSL, C_8-HSL, 3-OXO-C_8-HSL, 3-hydroxy-C_8-HSL	Root nodulation/symbionts, plasmid transfer, growth inhibition, stationary-phase adaptation
Rhodobacter sphaeroides	7-cis-C_{14}-HSL	Community escape
Serratia sp. ATCC39006	C_4-HSL, C_6-HSL	Antibiotic, pigments, exoenzymes
Serratia liquefaciens MG1	C_4-HSL, C_6-HSl	Swarming motility, exoprotease, biofilm development, biosurfactant
Vibrio fischeri	3-OXO-C_6-HSL	Bioluminescence
Vibrio harveyi	3-hydroxy-C_4-HSL	Bioluminescence
Yersinia enterocolitica	C_6-HSL, 3-OXO-C_6-HSL, 3-OXO-C^{10}-HSL, 3-OXO-C_{12}-HSL, 3-OXO-C_{14}-HSL	Swimming and swarming motility

open when the pH is raised from 1 to 2. By introducing a C3 acyl chain (C3-HSL), the ring remains largely intact at pH 2, but approximately 70% is hydrolyzed by pH 6, in contrast to C4-HSL, whose ring is completely opened only at pH 8 (Yates et al. 2002). Ring-opened AHLs are not active as quorum-sensing signal molecules. Given the stability of the HSL ring at acidic pH values, it is perhaps not too surpri sing that the acidophilic extremophile, *Acidithiobacillus ferrooxidans* employs AHL-dependent quorum sensing. This organism is involved in the bioleaching of metal sulphide ores and produces at least nine AHLs including *N*-acyl,

N-(3-oxoacyl), and *N*-(3-hydroxyacyl) compounds ranging from C8 to C16 in acyl chain length (Farah et al. 2005). The structures of various AHLs have been described in the literature (Williams 2007; Cooley et al. 2008; Hodgkinson et al. 2010).

6.4 QS-Regulated Bacterial Traits

Since the discovery of QS regulation in *V. fischeri,* numerous QS systems have been described in bacteria. They regulate diverse functions (Parsek and Greenberg 2005) such as production of antifungal or antibiotic compounds, motility patterns, virulence factors, biofilm formation, and plasmid conjugal transfer. In the case of *P. aeruginosa*, at least 6% of its genome is AHL-regulated via the las and rhl quorum-sensing systems (Hentzer et al. 2003; Wagner et al. 2004). LasI primarily directs the synthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL; Pearson et al. 1994) and together with the LasR regulates the production of virulence factors including elastase, LasA protease, alkaline protease, and exotoxin A (de Kievit and Iglewski 2000). RhlI directs the synthesis of C4-HSL (Winson et al. 1995), which activates RhlR and in turn induces, for example, the production of rhamnolipids, elastase, LasA protease, hydrogen cyanide, pyocyanin, siderophores, and LecA and LecB lectins (Winson et al. 1995; Latifi et al. 1996; Winzer et al. 2000; Diggle et al. 2002).

In *Chromobacterium violaceum, cviI* gene encodes for the enzyme of *N*-hexanoyl-L-homoserine lactone (HHL), which induces the production of violacein (purple pigment) and chitinase. In *C. violaceum* ATCC 31532, a number of phenotypes including production of violacein, hydrogen cyanide, and exoprotease are known to be QS-regulated (McClean et al. 1997).

luxRI homologues have been identified in *Burkholderia cepacia*, termed *cepR* and *cepI*. The CepRI quorum-sensing system has both positive and negative regulatory roles in *B. cepacia*, increasing protease production while simultaneously decreasing siderophore synthesis (Lewenza et al. 1999).

E. carotovora uses quorum sensing for exoenzyme production for successful tissue destruction and evasion of plant defenses (Pirhonen et al. 1993). This exoenzyme production is regulated by LuxI homologues ExpR and ExpI. *E. carotovora* quorum sensing is made even more complex by the finding that synthesis of the broad-spectrum antibiotic carbapenem is regulated using a second quorum-sensing system. Carbapenem production is regulated by CarR and CarI; the latter catalyzes the synthesis of 3-oxo-C6-HSL (Chhabra et al. 1993; McGowan et al. 1995).

A. tumefaciens produces 3-oxo-C8-HSL that stimulates plasmid conjugation (Zhang et al. 1993) together with a regulator called TraR, capable of activating expression of the *tra* genes (Piper et al. 1993), and this suggests that conjugal transfer in *A. tumefaciens* is regulated by a quorum-sensing system. Several other pathogens, such as *P. aeruginosa* (Brint and Ohman 1995), *A. hydrophila* (Chapon-Herve et al. 1997), and *S. marcescens* (Cheung et al. 1992) have been shown to regulate biofilm formation by QS. There are many bacterial species known to produce AHL signals, but the corresponding biological function remains to be unveiled. Several reports have

described the QS-regulated behavior and associated signal molecules in different bacterial systems (de Kievit and Iglewski 2000; Williams 2007; Asad and Opal 2008; Defoirdt et al. 2010). Some QS-regulated traits are provided in Table 6.1.

6.5 Isolation, Purification, and Characterization of AHL Molecules

AHLs can be extracted from spent supernatants of late-exponential phase cultures (Shaw et al. 1997; Schaefer et al. 2000). Briefly, bacteria are removed by centrifugation, supernatants are extracted twice with equal volumes of ethyl acetate, and the extracts are dried over anhydrous magnesium sulphate, filtered, and evaporated to dryness. Adapting the standard procedure, Khan et al. (2009) extracted AHL from *Chromobacterium violaceum* 31532 (an overproducing strain of C6-AHL). Bacterial culture is grown in 4 L of Luria broth on a shaking incubator at 28°C for 18 h and centrifuged at 12,000 × *g*. The supernatant is sterilized by membrane filtration (0.22 μm). The filtrate is extracted with acidified ethyl acetate (0.1 v/v acetic acid) (supernatant and acidified ethyl acetate, 7:3, v/v) and finally concentrated and dried by rotary evaporation at 40°C and reconstituted in acetonitrile.

6.6 Assays for AHL Detection

QS signal molecules are produced in very low concentrations with various interfering compounds (Brelles-Marino and Bedmar 2001; Van Houdt et al. 2007). Most of these detection methods focus on the existence of various AHLs in different bacterial cultures to demonstrate their stimulation actions in quorum-sensing mechanisms (Yan et al. 2007).

To date, many qualitative and quantitative approaches have been developed to detect AHLs. These include whole-cell-based bioassays using AHL-specific biosensors, thin-layer chromatography (TLC), gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), isotopic labeling, and absorbance-based assays (McClean et al. 1997; Zhu et al. 2003; Tait et al. 2005; Singh et al. 2006; Yang et al. 2006; Cataldi et al. 2007; Yan et al. 2007; Pinto et al. 2010; Wang et al. 2010).

6.6.1 Detection Through Bioassays

Numerous bioassays and sensor systems have been developed that allow facile detection, characterization, and quantification of microbial acyl HSLs (McClean et al. 1997; Zhu et al. 2003; Steindler and Venturi, 2007; Kawaguchi et al. 2008) Detection of acyl HSLs has been facilitated by the development of a variety of

bioassay strains. Such strains contain an easily assayable reporter gene and lack all AHL synthases such that reporter activity requires exogenous AHLs. The AHL system identified to date has been made possible mainly by the use of bacterial biosensors that are capable of detecting the presence of AHLs. The biosensors do not produce AHLs and contain a functional LuxR family protein cloned together with a cognate target promoter (usually *luxI* synthase), which positively regulates the transcription of a reporter gene (e.g., bioluminescence, β-galactosidase, green fluorescent protein, and violacein production). AHL biosensors have been constituted based on several LuxR family proteins that detect short and medium acyl chain AHLs (C4-C8), long acyl chain AHLs (C10-C12), and 3-hyroxy-AHLs (Steindler and Venturi, 2007). Various reporter genes have been employed, including *lacZ*, *gfp*, *lux,* and the production of an endogenous pigment.

Different reporter strains have been used in agar-plate-based bioassays; however, all methods are basically similar. First, the reporter strain is mixed with agar and then the test strain or extract is spotted on top of the agar or in wells. Following a sufficient period of incubation, the surroundings of the spots or wells are screened for the presence of the reporter gene product (for example, violacein in *C. violaceum*). However, this approach will not indicate if a sample contains multiple or only a single signal molecule. Detection can, however, be accomplished by thin-layer chromatography. Monitoring through T-streaks or assays of conditioned media, AHL biosensors greatly facilitate the characterization of quorum-sensing signal molecule(s) produced by a given organism. Other compounds produced by the target organism may give false-negative results in these assays because of bactericidal or bacteriostatic effects on the biosensor. The extraction of AHLs from spent culture medium using organic solvents (Shaw et al. 1997; Schaefer et al. 2000) can overcome this problem and also allows for concentration of any AHL present. Where possible, transformation of target organisms with biosensor plasmids can also circumvent the problem of antimicrobial activity (Zhu et al. 2003). Furthermore, the assay of reporter gene expression throughout growth in these transformed strains enables any cell-density-dependent production to be determined. Broad-host-range vectors such as pSB403, based upon pRK415, are best suited to this type of study (Winson et al. 1998a). AHL biosensors have also been used effectively to screen for recombinant clones of AHL synthase genes in *E. coli*. Genomic libraries prepared from organisms activating the biosensor can be introduced into an *E. coli* strain containing an AHL reporter plasmid, and the resulting transformants can be screened for reporter activation (Winson et al. 1998). Alternatively, patched libraries can be screened with biosensor overlays.

6.6.2 Chemical Detection

Thin-layer chromatography, gas chromatography, high-performance liquid chromatography (HPLC), and isotope labeling (Teplitski et al. 2000) are methods commonly used to analyze autoinducers. TLC coupled to a specific bioassay is an

effective technique that allows the testing of a great variety of isolates. It is useful for detection and quantification of autoinducers and to monitor their purification. AHLs can be purified by fractionating concentrated supernatant extracts using HPLC (Camara et al. 1998) or TLC (Shaw et al. 1997). Separation of supernatant with organic molecules (e.g., dichloromethane, ethyl acetate) is made on the basis of differences in mass and polarity.

TLC, coupled with a bioreporter, provides a simple and rapid technique for assessing the minimum number of different acyl-HSL species produced by a given organism (Shaw et al. 1997). Using TLC overlay procedures, fractionation and detection is possible within 24 h. Spots of pigmentation or bioluminescence can be imaged and compared with known standards on the basis of R_f. The technique also provides limited and preliminary information concerning the nature of these compounds present in extracts of the culture supernatants.

TLC also provides preliminary information on the number and nature of compounds present in the supernatant of bacterial cultures, R_f values calculated for the samples can be compared with those of the standards. Residual separation can be performed on C18 reversed-phase TLC plates (Shaw et al. 1997; Pinto et al. 2010). However, structures cannot be assigned based on chromatographic properties alone but rather can be assigned on the basis of spectroscopic properties. The most valuable tools for characterization are infrared spectroscopy, mass spectrometry, and nuclear magnetic resonance spectroscopy (Zhang et al., 1993; Debler et al., 2007; Lithgow et al. 2000; Donabedian 2003).

HPLC is an effective method for the fractionation and separation of AHLs for structural analysis. Biosensors can be used to identify active HPLC fractions, which can then be subjected to mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. The structure of the predicted molecule can then be confirmed by chemical synthesis (Chhabra et al. 1993; Wang et al. 2010).

A colorimetric assay developed by Yang et al. (2006) is a modified method of that reported by Goddu et al. (1955) for the analysis of ester molecules. This method is rapid, easy to use, and can analyze the quantity of lactone compounds and lactonase activity. Its detection limit is approximately 1 nmol for the lactone compounds, which is comparable to that of the HPLC method.

6.6.3 Application of Microbial and Chemical Assays

Biosensor-employing *Chromobacterium violaceum* was constructed by McClean et al. (1997), which produces and responds to C6-AHL. *C. violaceum* CVO26 strain is a violaceinand AHL-negative double mini Tn5 mutant in which a transposon is inserted into the *cviI* AHL synthase gene and another into a putative violacein repressor locus. Exposure of CVO26 strain to exogenous AHL results in rapid production of violacein. The most active agonist AHL for CVO26 is the natural *C. violaceum* AHL, i.e., C6-AHL. The other AHLs that cause reasonable induction are C6-3-oxo-AHL and C8-AHL (six times less active in comparison to C6-AHL),

C8-3-oxo-AHL (11 times less active), and C4-AHL (30 times less active). The strain responds very poorly to C4-3-oxo-AHL, and AHLs with an acyl chain length of C10 and longer have no activity. Further, not all 3-hydroxy-AHLs are detected by CVO26. This strain is well suited for detection on solid media via "T" streak analysis as well as TLC soft agar overlay technique.

Detection and quantification of long-chain AHLs, in particular C12-3-oxo-AHL, can be carried out with *P. aeruginosa* PAO1 M71LZ, a *lasI* genomic knockout mutant, which contains a transcriptional fusion of promoter of *rsaL* and reporter gene *lacZ* (Dong et al. 2005). The *rsaL* gene is directly regulated by the LasI/R AHL QS system (de Kievit et al. 1999; Rampioni et al. 2006). Providing C12-3-oxo-AHL to PAO1 M71LZ results in *rsaL* transcription via LasR, which is quantifiable through determination of β-galactosidase activity. This sensor is also employed for C10-3-oxo-AHL.

The *Agrobacterium tumefaciens*-based AHL biosensor detects a broad range of AHLs and also displays the greatest sensitivity toward these compounds (Cha et al. 1998; Farrand et al. 2002; Zhu et al. 2003). The biosensor consists of a three-plasmid system (pJZ384) (pJZ410) (pJZ372) in *A. tumefaciens* KYC55, which lacksTi plasmid and does not produce AHL. Plasmid pJZ384 contains TraR gene under the control of the phage T7 promoter, pJZ410 contains phage T7 RNA polymerase gene, and pJZ372 contains *traI–lacZ* reporter fusion. This biosensor has great sensitivity to a broad range of AHLs and can detect extremely low concentrations. Like other *A. tumefaciens*-based biosensors, it can also be used for TLC analysis. Extracts or supernatant can also be assayed directly and quantification can be determined using this biosensor strain (Zhu et al. 2003). The TraI/R AHL system is located in the Ti-plasmid and is well characterized, producing and responding to C8-3-oxo-AHL. AHL biosensor *A. tumefaciens* NT1 (pZLR4) consists of strain NT1 cured of Ti plasmid and thus unable to produce AHLs. The plasmid contains the *traR* gene, and one of the *tra* operons containing a *traG–lacZ* reporter fusion, whose transcription is regulated by the TraI/R AHL QS system, is responsible for Ti plasmid conjugal transfer (Cha et al. 1998; Farrand et al. 2002). It is sufficiently sensitive to many AHLs such that it requires only small amounts of AHL extracts (Farrand et al. 2002). The sensor is used by spotting colonies, supernatants, or sample extracts onto an overlay of the sensor grown in suitable medium containing X-gal. After overnight incubation, the presence of AHLs results in a blue zone around the site of application. Similar *A. tumefaciens* biosensors were constructed, namely, WCF47 (Zhu et al. 1998) and A136 (Fuqua and Winans, 1996) harboring a plasmid pCF218, which strongly expresses the AHL-responsive transcriptional factor TraR, and second a plasmid pCF372 carrying a TraR-regulated promoter *traI* transcriptionally fused to *lacZ*. This *A. tumefaciens* biosensor is very sensitive to a variety of AHLs ranging from C6–C14 AHL chains (Zhu et al. 1998; Mclean et al. 2004). A positive test for AHL production was indicated by blue coloration due to *lacZ* expression and X-gal hydrolysis in the biosensor (Fuqua and Winans 1996; Zhu et al. 1998; Mclean et al. 2004).

Several other biosensors have been developed that depend on a plasmid construct harboring the *luxCDABE* operon of *Photorhabdus luminescens* resulting in

bioluminescence as a reporter system (Winson et al. 1998). These plasmids are usually harbored in *Escherichia coli*, which does not produce AHLs. Plasmid pSB401 (Winson et al. 1998) and pHV200I^{-} (Pearson et al. 1994) are both based on the LuxR of *V. fischeri* and cognate *luxI* promoter controlling *luxCDABE* expression. The presence of AHLs, therefore, induces bioluminescence, which in a TLC analysis can be easily detected by exposing the TLC overlaid with biosensor to autoradiographic paper. They are most sensitive to cognate C6-3-oxo-AHL and display good sensitivity for C6-AHL, C8-3-oxo-AHL, and C8-AHL. These biosensors need photon camera equipment and, therefore, are not easy to use. These two LuxR biosensors, *E. coli* (pSB401) and *E. coli* (pHV200I^{-}), can be employed for the quantification of AHLs with the help of a luminometer (Winson et al. 1998). pSB403 contains the same arrangement of pSB401 (i.e., *luxR* and promoter of *luxI* controlling *luxCDABE* expression) cloned in a wide-host-range mobilizable plasmid, which provides the advantage that it can be harbored in several other Gram-negative bacteria (Winson et al. 1998). A biosensor sensitive for C4-AHL is *E. coli* (pSB536). The plasmid is constructed using *ahyR* of *Aeromonas hydrophila* and cognate *ahyI* gene promoter fused to *luxCDABE.* Similarly, *E. coli* (pAL101) is another plasmid-based sensor responding to C4-AHL. This construct is composed of *rhlR* and cognate promoter *rhlI* fused to *luxCDABE* (Lindsay and Ahmer 2005). The RhlI/R AHL QS system belongs to *Pseudomonas aeruginosa*, and C4-AHL is the cognate signal molecule. This plasmid-based sensor works best if harbored in *E. coli sdiA* gene mutant. SdiA is an orphan LuxR family protein present in *E. coli* that can activate the *rhlI* promoter, thus interfering with C4p-AHL detection (Lindsay and Ahmer 2005). *E. coli* does not have SdiA cognate LuxI family synthase and does not synthesize AHL.

Plasmid sensor pSB1075 contains *lasR* gene and cognate *lasI* gene promoter controlling *luxCDABE* expression (Winson et al. 1998). This plasmid can be harbored in *E. coli,* which responds well to C12-oxo-AHL, C10-oxo-AHL, and C12-AHL. Another *E. coli* plasmid sensor also based on the LuxI/R system is pKDT17 (Pearson et al. 1994). This plasmid contains *lasR* under the control of the *lac* promoter and a *lasB–lacZ* translational fusion; hence, response to exogenous AHL is detected via β-galactosidase activity. The *lasB* gene encodes for an elastase, regulated by the LasI/R AHL QS system. The *E. coli* (pKDT17) AHL biosensor responds strongly to C12-AHL, C10-AHL, and their derivatives; it does not detect any of the shorter and 3-hydroxy AHLs, however (Cha et al. 1998). Both biosensors can be used for the quantification of AHL.

To detect 3-hydroxy-AHLs, specific sensors have been developed based on the PhzI/R AHL QS system of *Pseudomonas flourescens* 2-79 (Khan et al. 2005). PhzI/R regulates the expression of the *phzABCDEFG* operon responsible for the biosynthesis of phenazine-1-carboxylate, an antimicrobial compound. PhzI of *P. fluorescens* is responsible for the production of six different AHLs of which the dominant and cognate signal is C6-3-hydroxy-AHL. The biosensor consists of two plasmid systems harbored in the wild-type *P. fluorescens* 1855 strain, which does not produce AHLs. Plasmid pSF105 harbors the *phzR* gene under the control of the *trc* promoter, and the other plasmid, pSF107, contains the *phzR–phzA* divergent

PhzR-regulated dual promoter region fused between oppositely oriented *uidA* and *lacZ* reporters, which are detectable by β-glucuronidase and β-galactosidase activity, respectively. Using the *phzA–lacZ* reporter in pSF107, the AHL sensor responds best to C6-3-hydroxy-AHL with tenfold less sensitivity to C8-3-hydroxy-AHL. This sensor can be used in TLC analysis, and quantification can be carried out by measuring either β-glucuronidase or β-galactosidase activity (Khan et al. 2005).

A novel plasmid (pEAL01) has been constructed and transformed into *Pseudomonas aeruginosa* QSC105 for the detection of a broad range of acyl-homoserine lactones through the induction of *lasB–lacZ* transcriptional fusion (Ling et al. 2009). Monitoring β-galactosidase activity from this bioassay showed that *P. aeruginosa* (pEAL01) could detect the presence of eight acyl-homoserine lactones tested at physiological concentrations (Bernier et al. 2008).

Lukas et al. (2008) used *Vibrio harveyi* strain BB170-autoinducer bioassay to detect quorum-sensing autoinducer-2 molecule (AI-2) in culture fluids of commensal intestinal bacteria. Culture fluids of *Bacteroides vulgatus*, *Clostridium proteoclasticum*, *Escherichia coli*, *Eubacterium rectale*, *Lachnospira multipara*, *Pseudobutyrivibrio ruminis*, *Roseburia intestinalis*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* contained AI-2-like molecules.

Recently, several authors have reported the use of different biosensor strains in the detection of signal molecules (Li et al. 2008, 2010; Tao et al. 2009; Lipasova et al. 2009; Savka et al. 2010; Zhao et al. 2010).

6.7 Interferences in Bacterial Quorum Sensing

Quorum sensing helps to coordinate community-based bacterial behavior, but it is not essential for bacterial survival; therefore, inhibition of QS only interrupts the desired phenotype. For example, inhibition of QS may attenuate virulence, reduce biofilm formation, and increase bacterial sensitivity to drug therapy, and in some cases, turning on quorum sensing at an early stage itself may attenuate bacterial virulence. Therefore, inhibition of bacterial quorum sensing may not bring about a universally beneficial effect. Also, inhibition is useful as in the production of adjuvants, since no bactericidal and/or bacteriostatic effects are necessarily produced. Furthermore, bacterial quorum sensing is not a singular event, as at least six QS pathways have been identified so far (Ni et al. 2009).

Different bacteria may use different pathways and autoinducers to regulate QS; for example, only Gram-negative bacteria use acylated homoserine lactones (AHL) as autoinducers, while only Gram-positive bacteria use autoinducing peptides (AIP) as autoinducers. However, autoinducer-2 (AI-2) mediates quorum sensing in both Gram-positive and Gram-negative bacteria. However, blocking QS has attracted scientists to a number of applications in controlling diseases in both humans and plants. On the other hand, blocking QS hopefully provides more information for exploring the exact contribution of such systems in bacterial cell–cell communication, along with traits and genes involved in such process.

There are several ways to inhibit quorum sensing in their respective pathway(s) as described by many workers (Defoirdt et al. 2004; Gonzalez and Keshavan 2006; Guoliang and Mingxia 2009; Ni et al. 2009):

1. Inhibition of signal molecule biosynthesis
2. Blocking signal transduction
   (a) Inhibition of autoinducer transport/secretion
   (b) Inhibition of targets downstream of receptor binding
   (c) Sequestration of autoinducers using, for example, antibodies against autoinducers
3. Chemical inactivation and biodegradation of signal molecules
   (a) Degradation of autoinducers using either enzymes (such as lactonases) or catalytic antibodies (abzymes)
4. Inhibition of receptor molecules
   (a) Using antibodies that "cover" and therefore block autoinducer receptors
   (b) Autoinducer receptor antagonism

These strategies can be applied to achieve inhibition in AIP-mediated QS (Gram-positive), AHL-mediated QS (Gram-negative), and AIP-2-mediated QS. However, our focus in this chapter is on inhibition of AHL-mediated QS.

6.7.1 Inhibition of AHL-Mediated QS

6.7.1.1 Inhibition of Signal Molecule Biosynthesis

Most Gram-negative bacteria use AHLs as signaling molecules in quorum sensing. Different bacteria could share the same AHL or use their own autoinducers (AIs). Knowledge about signal generation can be exploited to develop quorum-sensing inhibitor molecules that target AHL signal generation. Various analogues of SAM, such as *S*-adenosylhomocysteine, *S*-adenosylcysteine, and sinefungin, have been demonstrated to be potent inhibitors of AHL synthesis catalyzed by the *P. aeruginosa* RhlI protein (Hoang and Schweizer 1999). Synthesis of AHL compounds by a LuxI or its homologue is the critical first step in the AHL-mediated quorum-sensing process. *S*-adenosylmethionine (SAM) is the precursor of AHL; therefore, inhibitors of SAM-utilizing enzymes can interfere with AHL-mediated quorum sensing.

Parsek et al. (1999) reported that analogues of *S*-adenosylmethionine (such as *S*-adenosylcysteine) inhibited activity of the *Pseudomonas aeruginosa LuxI* homologue RhlI by up to 97%. Since no AHL synthase sequence motifs were found in other enzymes with *S*-adenosylmethionine binding sites, it is possible to use the *S*-adenosylmethionine analogues as specific quorum-sensing inhibitors without affecting other vital processes in prokaryotic or eukaryotic organisms.

Lesic et al. (2007) identified halogenated AA analogues that specifically inhibited HAQ biosynthesis and disrupted MvfR-dependent gene expression.

These compounds restricted *P. aeruginosa* systemic dissemination and mortality in mice without perturbing bacterial viability, and inhibited osmoprotection, a widespread bacterial function. The *Pseudomonas aeruginosa* MvfR-dependent QS regulatory pathway controls the expression of key virulence genes and is activated via the extracellular signals 4-hydroxy-2-heptylquinoline (HHQ) and 3,4-dihydroxy-2-heptylquinoline (PQS), whose synthesis depend on anthranilic acid (AA), the primary precursor of 4-hydroxy-2-alkylquinolines (HAQs). These compounds provide a starting point for the design and development of selective anti-infectives that restrict human *P. aeruginosa* pathogenesis and possibly other clinically significant pathogens.

6.7.1.2 Blocking Signal Transduction

Blocking of quorum-sensing signal transduction can be achieved by an antagonist molecule capable of competing or interfering with the native AHL signal for binding to the LuxR-type receptor. Competitive inhibitors would conceivably be structurally similar to the native AHL signal to bind to and occupy the AHL-binding site but fail to activate the LuxR-type receptor. Noncompetitive inhibitors may show little or no structural similarity to AHL signals, as these molecules bind to different sites on the receptor protein. Several reports describe the in vitro application of AHL analogues to achieve inhibition of the quorum-sensing circuits of various bacteria (Zhu et al. 1998; Parsek et al. 1999; Rasmussen and Givskov 2006).

Synthetic Analogues for Quorum Sensing Autoinducers

A variety of structural analogues for different AHL molecules have been studied for their effects on the quorum-sensing system of the related bacterial strain. A study on the analogues of 3-oxo-C8-HSL shows that the nature of the agonistic or antagonistic activity strongly depends on the expression of the TraR protein (Zhu et al. 1998; Parsek et al. 1999). Overexpression of the response regulator, as seen in the case of several reporter strains, results in recognition of most AHL analogues as agonists. In the study of Gonzalez and Keshavan (2006), wild-type levels of TraR identified 3-oxo-C7-HSL, 3-oxo-C11-HSL, and 3-oxo-C12-HSL as agonists. Most of the 33 compounds tested inhibited AHL-dependent gene expression. C8-HSL, 3-oxo-C6-HSL, C7-HSL, C10-HSL, and 3-OH-C9-HSL were identified as the most effective antagonists. Overall, the agonistic or antagonistic activity, each requiring binding to TraR, is effective only when the acyl chain lengths are closer to that of the cognate AHL, 3-oxo-C8-HSL. When TraR was overexpressed, the bacterium was more sensitive to low concentrations of 3-oxo-C8-HSL than its parent strain (Gonzalez and Keshavan 2006).

Similar studies conducted with synthetic analogues for cognate AHLs of other quorum-sensing systems indicate that the homoserine lactone ring is very important for biological activity, while the nature of the acyl chains was not that critical

for binding the response regulator (Reverchon et al. 2002). Olsen et al. (2002) synthesized AHL analogues based on the strategy that modifying the conserved lactone head group of AHLs will probably result in antagonistic molecules with a broader application range. Based on their observations, they concluded that the analogues with C-4 substitutions on the lactone ring were weak activators, implying that this part of the molecule is crucial for recognition by LuxR, whereas substitution at the third position on the lactone ring resulted in activators of LuxR. Two compounds containing carbamate lactones were identified as inhibitors, although they appeared to be less efficient than a furanone. Another study aimed at synthesizing analogues to 3-oxo-C6-HSL and C6-HSL with either ramified cycloalkyl or ramified aryl substituents at the C-4 position of the acyl chain. The authors concluded that the inducing activity is retained if one branch is introduced at the C-5 position of the acyl chain. The best antagonists were compounds with a phenyl group or a phenyl bearing a heteroatom in the *para* position. Naphthyl and biphenyl compounds showed no activity, probably due to steric hindrance. They also observed that the 3-oxo group, which is important for the inducing activity, also favors the antagonistic activity of phenyl derivatives. Overall, the secondary alkyl derivatives had agonistic activity, while the aryl and tertiary alkyl derivatives had antagonistic activity when tested with an *E. coli*-based luminescent biosensor strain containing a plasmid with the *lux* genes from *V. fischeri* (Gonzalez and Keshavan 2006).

Kelly et al. (2009) synthesized an analogue for the CAI-1 autoinducer in *Vibrio cholerae*, the bacterium that causes cholera. Control of virulence factor production and biofilm development is dependent on response to two extracellular quorum-sensing molecules, i.e., autoinducers. The strongest autoinducer, termed CAI-1 (for cholera autoinducer-1), was previously identified as (*S*)-3-hydroxytridecan-4-one. Biosynthesis of CAI-1 requires the enzyme CqsA. These workers determined the CqsA reaction mechanism, identified the CqsA substrates as (*S*)-2-aminobutyrate and decanoyl coenzyme A, and demonstrated that the product of the reaction is 3-aminotridecan-4-one, dubbed amino-CAI-1. CqsA produced amino-CAI-1 by a pyridoxal phosphate-dependent acyl-CoA transferase reaction. Amino-CAI-1 is converted into CAI-1 in a subsequent step via a CqsA-independent mechanism. They further found that cells release ≥100 times more CAI-1 than amino-CAI-1. Nonetheless, *V. cholerae* responds to amino-CAI-1 as well as CAI-1, whereas other CAI-1 variants do not elicit a quorum-sensing response. Thus, both CAI-1 and amino-CAI-1 have potential as lead molecules in the development of an anti-cholera treatment.

Recently, Chan et al. (2010) have identified the *aiiA* homologue, encoding an autoinducer inactivation enzyme catalyzing the degradation of *N*-acylhomoserine lactones of KM1S, a bacterial strain isolated from a Malaysian rainforest soil sample. A defined enrichment medium was used that specifically facilitated selection of quorum-quenching bacteria, which were amplified and cloned. Sequence analysis indicated the presence of the motif $_{106}$HXDH-59 amino acids-H_{169}-21 amino acids-D_{191} for *N*-acylhomoserine lactone lactonases. It degraded *N*-3-oxo-hexanoyl homoserine lactone and *N*-3-oxo-octanoyl homoserine lactone in vitro

rapidly at 4.98 and 6.56 μg AHL h^{-1} per 10^9 CFU/ml, respectively, as determined by the Rapid Resolution Liquid Chromatography.

These studies have generated substantial knowledge about the structure–function relationships of AHL signals, which is of great value for the continued search for potent quorum-sensing inhibitors. Such AHL antagonists can be synthesized and are classified on the basis of whether the structural modification was on (1) the lactone ring, (2) the acyl side chain, or (3) both the acyl side chain and lactone ring.

Modification of the Acyl Side Chain

As mentioned earlier, different AHLs are functional in different bacteria. Because of the structural diversity in AHLs, there are also different receptors/regulators that respond in a specific fashion to specific AHLs. For example, AhyR exists in *A. hydrophila*, Rhl and LasR in *P. aeruginosa*, SwrR in *S. liquefaciens*, CviR in *C. violaceum*, LuxR in *V. fischeri*, LuxN in *V. harveyi*, TraR in *A. tumefaciens,* and VanR in *V. anguillarum* (Whitehead et al. 2001). Some AHLs can be agonists in certain bacterial species and strains but serve as antagonists in others. One interesting observation in AHL-mediated quorum sensing is that it is common for AHLs with long side chains to antagonize the function of AHLs with short side chains.

Acyl side chain modified AHL analogues were mostly studied for their ability to antagonize the effect of natural ligands through receptor binding in bacteria such as *V. fischeri* (Reverchon et al. 2002), *P. aeruginosa* (Kline et al. 1999), and *A. tumefaciens* (Zhu et al. 1998). It has been found that most quorum-sensing receptors respond to analogues that differ from the natural ligands by only two carbons. Too many changes could eliminate their agonist effect. It has also been found that the homoserine lactone ring is very important to the activities of these analogues compared with the acyl chain (Schaefer et al. 1996). Interestingly, AHL analogues with a longer side chain than the native AHL generally appear to be more efficient inhibitors than AHL analogues with a shorter side chain.

In one study of quorum sensing in *E. carotovora*, it was reported that increasing the length of the acyl side chain by one methylene unit reduced activity by 50%, whereas a two unit extension reduced activity by 90%. Decreasing the chain length by one methylene unit decreased activity to 10% (Chhabra et al. 1993). A study investigating the *P. aeruginosa* LasR receptor suggested that the fully extended chain geometry is necessary for activation, whereas constrained analogues locked into different conformations showed no activity (Kline et al. 1999).

Recently, Bokhove et al. (2010) have reported the first crystal structure of an AHL amidohydrolase, the AHL acylase PvdQ from *Pseudomonas aeruginosa*. PvdQ has a typical α/β heterodimeric Ntn-hydrolase fold, similar to penicillin G acylase and cephalosporin acylase. However, it has a distinct, unusually large, hydrophobic binding pocket, ideally suited to recognize C12-fatty-acid-like chains of AHLs. Binding of a C12 fatty acid or a 3-oxo-C12 fatty acid induces subtle conformational changes to accommodate the aliphatic chain.

Modification of the Lactone Ring

The homoserine lactone moiety is generally very sensitive to modifications, and the chirality is crucial to biological activity. Natural AHL signals are L-isomers, whereas D-isomers are generally devoid of biological activity (Chhabra et al. 1993). The acyl side chain appears essential for activity, as exemplified in *E. carotovora*, where the unsubstituted homoserine lactone ring fails to activate the quorum-sensing system. Conversion of the homoserine lactone ring to a homoserine lactame ring results in a molecule without agonistic or antagonistic properties. A change of the homoserine lactone structure to a homoserine thiolactone ring appears permissible in several quorum-sensing systems (Schaefer et al. 1996). A recent study has showed that LasR and RhlR proteins responded differently to changes in the homoserine lactone moiety (Smith et al. 2003). This may indicate that the two *P. aeruginosa* AHL receptors differ significantly in their AHL binding sites.

As mentioned earlier, the quorum-sensing system in *P. aeruginosa* is controlled by two distinct yet interrelated pathways (Pearson et al. 1997), the las and rhl systems. There are two receptor proteins, LasR and RhlI, and two autoinducers (PAI1 and PAI2) belonging to the AI-1 family. Smith et al. (2003) synthesized a library of analogues of *P. aeruginosa* autoinducers PAI1 and PAI2, in which the respective 3-oxo-C12 and the C4 side chains were retained and the lactone portion was changed to different amines, alcohols, and ketones. The compounds were tested using the PAO-JP2 strain of *P. aeruginosa* in which both AI synthase genes, *lasI* and *rhlI*, were removed (Pearson et al. 1997).

Recently, Chen et al. (2010) have isolated the AHL-lactonase ($AiiA_{B546}$) from *Bacillus* sp. B546 that was produced extracellularly in *P. pastoris* with a yield of 3,558.4±81.3 U/mL in a 3.7-L fermenter when using 3-oxo-C8-HSL as the substrate.

Simultaneous Modifications on Both the Lactone Ring and Side Chain

Thomas et al. (2006) studied more quorum-sensing agonists/antagonists by understanding the immunomodulatory effects of AHL analogues in pathogenic bacterium *P. aeruginosa* and synthesized four AHL analogues. Instead of changing only the acyl chain, the lactone head group was changed to a 2-pyridyl analogue (Welch et al. 2000, a LasR antagonist) and to nonhydrolyzable cyclic ketones (Zhu and Winans 2001).

6.7.1.3 Chemical Inactivation and Biodegradation of Signal Molecules

Bacterial cell-to-cell communication can be inhibited by a decrease in the active signal-molecule concentration in the environment. AHL decay might be a consequence of a nonenzymatic reaction, e.g., AHL signals are subject to alkaline hydrolysis at high pH values (Yates et al. 2002). Some bacteria have been reported to specifically degrade AHL signals (Dong et al. 2000; Leadbetter and Greenberg 2000).

Chemical Inactivation

AHL could be chemically inactivated via alkaline hydrolysis, such as through oxidized halogen antimicrobials. These antimicrobials, at a concentration of approximately 0.14 mmol L^{-1}, were found to decrease the concentration of 3-oxo-substituted AHL to about one fourth after 1-min incubation but had no effect on unsubstituted ones. Moreover, the inactivation of 3-oxo-substituted AHL was shown to proceed in the presence of polysaccharide biofilm compounds despite the much higher concentration of the latter compared to the AHL concentration (Borchardt et al. 2001; Francesco et al. 2007).

Biodegradation

Some bacteria block the quorum-sensing systems of their bacterial competitors to obtain a selective advantage. The actual inactivation of the signal compound can be mediated by two types of enzymes, namely, AHL lactonases and AHL acylases. Further, research has demonstrated that genes encoding AHL-degrading lactonases are widespread in many *Bacillus* species (Lee et al. 2002; Dong et al. 2004). These AiiA homologues showed about 90% sequence homology at the amino-acid level. Dong et al. (2000) were the first to highlight that enzymatic AHL inactivation could be used as a biocontrol strategy and found a *Bacillus* species strain 240B1, which produced an enzyme termed AiiA that catalyzed the hydrolysis of AHL molecules. The purified enzyme, at a concentration of 50 mg L^{-1}, reduced the concentration of *N*-(3-oxohexanoyl)-l-homoserine lactone from 20 μmol L^{-1} to about 5 μmol L^{-1} after 10 min (Dong et al. 2001; Park et al. 2007).

Expression of the *aiiA* gene in the plant pathogen *Erwinia carotovora* resulted in reduced release of AHL signals, decreased extracellular pectolytic enzyme activity, and attenuated soft rot disease symptoms in all plants tested (Dong et al. 2000). Moreover, transgenic plants expressing AiiA have been shown to be significantly less susceptible to infection by *E. carotovora* (Dong et al. 2001). In another study, Leadbetter and Greenberg (2000) isolated a strain *Variovorax paradoxus* VAI-C from a soil sample and demonstrated that *V. paradoxus* cleaves the AHL by an AHL acylase enzyme, releasing homoserine lactone and a fatty acid. The *V. paradoxus* strain is able to grow using 3-oxo-C6-*N* homoserine lactone as the sole energy and nitrogen source. Molina et al. (2003) also tested the efficacy of using an AHL-degrading *Bacillus* sp. strain for the biocontrol of plant diseases. Lin et al. (2003) isolated an AHL-inactivating bacterium, *Ralstonia sp.* Strain XJ12B, from a mixed-species biofilm. The enzyme responsible for the AHL-inactivating activity (AiiD) was purified, and subsequently, *N*-(3-oxodecanoyl)-l-homoserine lactone was incubated with the purified enzyme. Electrospray ionization-mass spectrometry of the hydrolysis product demonstrated that the AiiD enzyme hydrolyzes the amide bond of AHLs. Moreover, Xu et al. (2003) investigated the ability of a eukaryotic counterpart of these bacterial enzymes to inactivate AHL molecules. Different AHLs were shown to be inactivated by the porcine kidney acylase I enzyme. Since the

Table 6.2 Occurrence of AHL degradation enzymes in prokaryotes and eukaryotes [adapted from Dong and Zhang (2005)]

Species	Genes	Enzymes
Prokaryotes		
Bacillus sp.240B1	*aiiA*	AHL Lactonase
B. thuringiensis	*aiiA* homologues	AHL Lactonase
B. cereus	*aiiA* homologues	AHL Lactonase
Agrobacterium tumefaciens	*attM, aiiB*	AHL Lactonase
Arthrobacter sp.IBN110	*ahlD*	AHL Lactonase
Klebsiella pneumoniae	*ahlK*	AHL Lactonase
Ralstonia strain XJ12B	*aiiD*	AHL Acylase
Pseudomonas strain PAI-A	*pvdQ*	AhL Acylase
P. aeruginosa PAO1	*pvdQ*	AHL Acylase
Eukaryotes		
Human (airway epithelia)	*PON*s	Lactonase
Porcine (kidney)	*ACY1*	Acylase I

inactivation was greatest at high pH values, the effect could have been due to simple alkaline hydrolysis of the lactone ring.

A list of AHL-degrading enzymes produced by prokaryotes and eukaryotes is given in Table 6.2. More recently, other authors have reported production of chemicals by bacteria that disrupt the QS system in other bacterial cells (Lesic et al. 2007; Chan et al. 2010; Chen et al. 2010).

6.7.2 *Inhibition of Other Quorum-Sensing Systems*

Mechanisms similar to inhibition in AHL-mediated QS have been reported by various workers in AI-2-mediated and also in AIP-mediated QS systems (Ni et al. 2009). In brief, inhibitors targeting histidine kinase or antagonists that target the AIP receptor have been described for inhibition of QS among Gram-positive bacteria (Wright et al. 2004). Inhibitors targeting synthesis of AI-2 have been reported by several workers (Zhu et al. 2004; Alfaro et al. 2004; Singh et al. 2006). Inhibition has also been achieved by blocking the AI-2 receptor and by the use of AI-2 antagonists (Niu et al. 2006; Ni et al. 2008, 2009).

6.7.3 *Quorum-Sensing Inhibitors Expressed by Higher Organisms*

A number of reports describe the ability of higher organisms to interfere with AHL-mediated quorum sensing. Several workers have found that plant and animal hosts produce quorum-sensing antagonists that can bind to quorum-sensing response regulators but fail to activate them (Defoirdt et al. 2004; Gonzalez and Keshavan

2006). Many plants and fungi have co-evolved and established carefully regulated symbiotic associations with bacteria. Many plant-associated proteobacteria possess AHL-mediated quorum-sensing systems (Cha et al. 1998; Manefield and Turner 2002). Importantly, both plants and fungi are devoid of the active immune systems that are observed in mammals; rather, they rely on chemical defense systems to respond to bacteria in the environment. For these reasons, it might be expected that plants and fungi have evolved to produce chemical compounds to inhibit (or in other cases to stimulate) bacterial AHL-mediated communication.

6.7.3.1 Inhibition of QS by Halogenated Furanone Compounds

The ability of bacteria to form biofilms is a major challenge for organisms at risk of infection, such as humans, other animals, and marine eukaryotes. Marine plants in the absence of an advanced immune system are prone to disease (Fenical 1997). Bacteria can be highly detrimental to marine algae and other eukaryotes (Littler and Littler 1995). The best-characterized example is that of the Australian red marine macroalga *Delisea pulchra*. It has developed a defense mechanism to protect itself from extensive bacterial colonization (Givskov et al. 1996). The alga produces a range of halogenated furanones [(5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanones] as antagonists for AHL-mediated quorum sensing and display antifouling and antimicrobial properties (de Nys et al. 1996). The halogenated furanones most probably bind to *LuxR* family proteins without activating them (Manefield et al. 1999; Rasmussen et al. 2000). This particular alga originally attracted the attention of marine biologists because it was devoid of surface colonization, i.e., biofouling, unlike other plants in the same environment. Biofouling is primarily caused by marine invertebrates and plants, but bacterial biofilms are believed to be the first colonizers of submerged surfaces, providing an initial conditioning biofilm to which other marine organisms may attach (Rice et al. 1999). Therefore, the abundance and composition of the bacterial community on the surface will significantly affect the subsequent development of a macrofouling community (Belas 2003). Extensive experimental evidence in support of this model has accumulated in recent years. This includes the observations that furanones (a) repress AHL-dependent expression of *V. fischeri* bioluminescence (Manefield et al. 1999), (b) inhibit AHL-controlled virulence factor production and pathogenesis in *P. aeruginosa* (Hentzer et al. 2003), (c) inhibit quorum-sensing-controlled luminescence and virulence of the black tiger prawn pathogen *Vibrio harveyi* (Manefield et al. 2000), and (d) inhibit quorum-sensing-controlled virulence of *E. carotovora* (Manefield et al. 2001). The natural furanone compounds have little or no effect on the quorum-sensing systems of *P. aeruginosa*. The furanone-repressed genes include many previously known as quorum-sensing-regulated genes, including numerous *P. aeruginosa* virulence factor genes such as *lasB* (encoding elastase), *lasA* (encoding LasA protease), *rhlAB* operon (regulating rhamnolipid production), *phzA-G* operon (encoding phenazine biosynthesis), *hcnABC* operon (regulating hydrogen cyanide production), and *chiC* (encoding chitinase).

Ren et al. (2001) found that the natural furanone compound (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone could also inhibit the AI-2-mediated quorum sensing in *V. harveyi* and *E. coli.* Defoirdt et al. (2007) studied the molecular mechanism of action and found that this furanone at 323 mM blocks three quorum-sensing pathways, AI-1, AI-2, and CAI-1, in *V. harveyi* by decreasing the DNA-binding activity of the master transcriptional regulator protein LuxRvh, which is the protein downstream of all the three quorum-sensing systems in *V. harveyi*.

It is notable that the synthetic furanones, in concentrations that significantly lower quorum-sensing-controlled gene expression in planktonic cells, were equally active against biofilm bacteria, despite the profoundly different modes of growth. Several furanone-based structural analogues have been synthesized and analyzed for their QSI activity. A recent study has reported the isolation of two natural products from a marine sponge and a *Pseudomonas* sp. that were structurally similar to furanones. These compounds, isocladospolide and acaterin, were used as templates for further modifications, and the resulting compounds were tested against LuxR-based *E. coli* biosensor strains. The 5H-furan-2-ones substituted with short alkyl chains were, in general, more antagonistic than the longer alkyl chain counterparts. Also, a substitution at the C-3 or C-5 position of the alkyl chain resulted in the most active antagonists (de Nys et al. 1996).

Synthetic furanones were initially tested on mouse lungs infected with *E. coli* strains carrying *luxR–pluxI–gfp*-based quorum-sensing sensors. AHL-dependent GFP expression was completely inhibited by intravenous injection of the active furanones. This inhibition was overcome by providing excess amounts of AHLs. This suggested that the synthetic furanones were transported via the blood to the lungs, entered lung tissue, and inhibited 3-oxo-C6-HSL-dependent gene expression in the bacteria. The death of mice inoculated with wild-type *P. aeruginosa* PAO1 was significantly delayed when they were treated with synthetic furanone, although the furanone could not prevent the death of the mice. In addition, there were fewer CFU of the bacteria on the furanone-treated lung surfaces compared to untreated controls. This suggests that the ability of the bacteria to colonize may be greatly reduced owing to inhibition of quorum sensing or because bacterial clearance from the lungs was enhanced due to furanones (Wu et al. 2004). A *C. violaceum*-based screen for QSI compounds used natural and chemically synthesized furanones and aimed at identifying compounds that either inhibited or enhanced quorum-sensing-dependent behavior. The authors designed a microtiter-dish-based assay that differentiated between compounds that affect growth, activate quorum sensing in *C. violaceum* strain CVO26, inhibit violacein formation induced by the cognate AHL, or enhance violacein formation in the presence of AHL. They found that some furanones were toxic at a higher concentration but inhibited quorum sensing at a much lower, nontoxic concentration. Several compounds were found to enhance quorum sensing at suboptimal C6 HSL concentrations but were antagonistic when optimal concentrations of C6 HSL were present. This study reflects on the variability of the activity of a given compound

based on its concentration as well as the availability of quorum-sensing-activating AHLs (Martinelli et al. 2004).

6.7.3.2 Inhibition of QS by Plant Products

It is known that exudates from higher plants such as peas, rice, soybeans, tomatoes, crown vetch, and *Medicago truncatula* (Barrel clover) also influence AHL-mediated quorum sensing. Reverse-phase high-performance liquid chromatography revealed that there are several different AHL-mimicking substances present in extracts from pea and *M. truncatula* seedlings (Gao et al. 2003). These plants secrete substances that stimulate AHL-dependent quorum sensing as well as substances that inhibit such responses. Recently, similar results have been obtained for microalgae (Teplitski et al. 2004; Francesco et al. 2007). The algae *Chlamydomonas reinhardtii*, *Chlamydomonas mutablis*, *Chlorella vulgaris*, and *Chlorella fusca* all stimulated quorum-sensing-regulated luminescence in wild-type *V. harveyi.*

Recently, another example of eukaryotic interference with AHL-mediated signaling has been provided by Teplitski et al. (2000), who showed that several plants secrete substances that mimic bacterial AHL signal activities and affect quorum-sensing-regulated behaviors in associated bacteria. Exudates from pea (*Pisum sativum*) were demonstrated to exhibit several distinct activities that either stimulated or inhibited bacterial AHL-dependent phenotypes.

In a study by Adonizio et al. (2006), out of 50 medicinal plants from southern Florida screened for anti-QS activity, six showed QS inhibition: *Conocarpus erectus* L. (Combretaceae), *Chamaecyce hypericifolia* (L.) Millsp. (Euphorbiaceae), *Callistemon viminalis* (Sol. ex Gaertn.) G. Don (Myrtaceae), *Bucida burceras* L. (Combretaceae), *Tetrazygia bicolor* (Mill.) Cogn. (Melastomataceae), and *Quercus virginiana* Mill. (Fagaceae). This study introduced not only a new mode of action and possible validation for traditional plant use but also a potentially new therapeutic direction for the treatment of bacterial infections.

Canavanine exuded from alfalfa seeds has been shown to possess the potential to affect the population biology of *Bacillus cereus* (Emmert et al. 1998). L-Canavanine is incorporated in the place of L-arginine into nascent protein chains during synthesis, resulting in altered protein structure and function and eventually leading to death of the targeted cell (Bence and Crooks 2003).

L-Canavanine is an arginine analogue found exclusively in the seeds of legumes. It has been reported to be as abundant as up to 5% (dry weight) of some leguminous seeds. In addition to serving as a nitrogen source for the germinating seedlings, L-canavanine is also known to serve as an allelopathic substance by inhibiting the growth of certain bacteria and phytophagous insects (Gonzalez and Keshavan 2006).

Recently, Khan et al. (2009) have revealed anti-QS activity of clove oil. They showed inhibition of QS-linked behavior in *C. violaceum* 12472, *C. violaceum* O26, and *P. aeruginosa* PAO1.

6.7.4 *Practical Significance of Bacterial QS Modulation in the Environment/Agriculture*

6.7.4.1 Roles of AHL-Degradation Enzymes in Host

AHL lactonases and AHL acylases are of microbial origins and were originally identified because of their activity against AHL signals (Leadbetter and Greenberg 2000; Lin et al. 2003). Studies have focused on their roles in microbe–microbe interactions and microbial physiology. It has recently been shown that an AHL lactonase producing strain of *B. thuringiensis* suppresses the QS-dependent virulence of the plant bacterial pathogen *E. carotovora* through a new form of microbial antagonism, signal interference (Dong et al. 2004). *E. carotovora* produces and responds to AHL signals to regulate the antibiotic production and expression of virulence genes; such QS-synchronized functions could be of critical importance for the pathogen in competing for ecological niches in microbe–microbe competition and pathogen–host interactions. Similarly, the expression of AHL lactonase in isolates of the soil bacterium *P. fluorescens* produces a similar effect in the biocontrol of *E. carotovora* (Molina et al. 2003). These data clearly indicate that AHL lactonase plays a significant role in obtaining competitive advantages for its producer over that of its competitors in natural ecosystems.

The findings that PON enzymes could degrade AHL signals (Chun et al. 2004) suggest that these generic hydrolytic enzymes may also contribute to defense against pathogenic invaders. Characterization of their specificity and efficiency in AHL degradation, as well as their expression pattern, would allow for a thorough assessment of their roles in pathogen–host interactions.

6.7.4.2 Biotechnological and Pharmaceutical Implications of AHL Degradation Enzymes

Because bacterial quorum sensing is implicated in various pathologically relevant events, it is conceivable that inhibitors of bacterial quorum sensing could have therapeutic applications. Given that QS-deficient mutants of bacterial pathogens are defective in virulence gene expression and become avirulent, it might be possible to control bacterial infections by quenching the QS signaling of microbial pathogens (Jiang and Su 2009; Choudhary and Dannert 2010). The discovery of quorum-quenching enzymes, in addition to quorum-sensing inhibitors (Zhang and Dong 2004), has provided essential tools to assess the feasibility of this novel strategy. The expression of a quorum-quenching enzyme, regardless of an AHL lactonase or AHL acylase, either in the plant or human pathogens *E. carotovora* and *P. aeruginosa,* respectively, significantly reduces their virulence (Lin et al. 2003; Molina et al. 2003).

Transgenic plants expressing AHL-lactonase can effectively quench bacterial QS signaling and disintegrate bacterial population-density-dependent infections, whereas untransformed control plants develop severe disease symptoms

(Dong et al. 2001). These results demonstrate that externally expressed AHL degradation enzyme is sufficient in eliminating the QS signals of physiologically relevant concentrations and in suppressing the QS-dependent virulence gene expression by pathogens. As the constitutive expression of disease-resistant "R" genes might accompany severe yield and biomass penalties, the integration of quorum-quenching mechanisms with the inducible plant defense systems could be the most rational way to build proactive host defense mechanisms against pathogenic invaders (Zhang 2003). Therefore, the genes encoding these novel quorum-quenching enzymes might hold great promise for the genetic engineering of plant disease resistance.

Quorum-quenching enzymes could also be explored as a new version of antagonism for the biocontrol of microbial infections. Several natural or engineered AHL lactonase-producing strains, including *B. thuringiensis*, *Arthrobacter* sp., and *P. fluorescens*, significantly reduced potato soft rot when coinoculated with the pathogen *E. carotovora,* which otherwise causes severe soft rot disease symptoms (Molina et al. 2003; Dong et al. 2004). Antibiotic production has been the major mechanism of microbial antagonism commonly exploited in the biocontrol of bacterial and fungal diseases. The finding that QS could be a widely conserved mechanism in the regulation of virulence suggests that quorum-quenching mechanisms might have promising potentials in biocontrol. Furthermore, several authors have reported successful inhibition of virulence by interrupting QS in pathogens (Lesic et al. 2007; Kelly et al. 2009; Jiang and Su 2009; Chen et al. 2010; Sintim et al. 2010).

6.7.4.3 Transgenic Plants

Quorum sensing appears to be crucial for plant-bacterial interactions, be they pathogenesis or symbiosis (Gonzalez and Marketon 2003). A timely activation of specific phenotypes responsible for interaction with the host plant ensures a successful establishment of a bacterial population on a host. A premature activation of these bacterial phenotypes could trigger early defense responses and, therefore, may be deleterious to the bacterial population (Zhang 2003). This concept has been exploited to create transgenic plants encoding bacterial AHL synthases such that the plants are now capable of producing AHL signal molecules. Fray et al. (1999) cloned the *yenI* AHL synthase from *Yersinia enterocolitica* and targeted it to the chloroplasts of tobacco plants to create transgenics that produced 3-oxo-C6-HSL and C6-HSL. Another report involving the cloning of the *expI* gene from *E. carotovora*, which is responsible for the synthesis of 3-oxo-C6-HSL, into tobacco showed that the transgenic plants produced the active signal molecule and exhibited enhanced resistance to infection by wild-type *E. carotovora*.

Cloning of a bacterial AHL lactonase enzyme (AiiA from *Bacillus* sp. strain 2401B) into tobacco and potato plants by Dong et al. (2001) illustrates a potentially effective method to control bacterial infections. Soluble protein extracted from transgenic tobacco leaves and potato tubers inactivated 3-oxo-C6-HSL activity in vitro.

Furthermore, transgenic potato plants containing the *yenI* gene shows increased susceptibility to soft rot infections by *Erwinia* strains. It was shown that the degree of susceptibility of transgenic potato plants to soft rot varied depending on the tissue tested and on the strain of *E. carotovora* (Gonzalez and Keshavan 2006). The contrasting results of cloning AHL synthase into tobacco and potato plants, with one showing increased resistance and the other enhanced susceptibility to the pathogens belonging to the same genus, *Erwinia*, show that further research on evaluating the mechanism of action along with elucidating the details of bacterial pathogenesis is necessary.

Recently, Vanjildorj et al. (2009) have developed a transgenic Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) inbred line, Kenshin, with high tolerance to soft rot disease. The tolerance was conferred by expression of *N*-acyl-homoserine lactonase (AHL-lactonase) in Chinese cabbage through an efficient *Agrobacterium*-mediated transformation method. To synthesize and express the AHL-lactonase in Chinese cabbage, the plant was transformed with the *aii* gene (AHL-lactonase gene from *Bacillus* sp. GH02) fused to the PinII signal peptide (protease inhibitor II from potato). Transgenic plants showed a significantly enhanced tolerance (two- to threefold) to soft rot disease compared to wild-type plants. Thus, expression of the fusion gene *pin*IISP–*aii* reduces susceptibility to soft rot disease in Chinese cabbage.

6.8 Conclusion

It appears that quorum sensing is a common regulatory mechanism among bacteria. In plant-associated bacteria, quorum sensing is used to control a broad range of traits, as found in human pathogenic bacteria. Under natural conditions, bacteria must compete with complex communities of other microorganisms to colonize and persist on plants and other hosts. Competitiveness and survival of bacteria in soil and rhizosphere may be influenced by the expression of traits controlled by quorum sensing. This type of relationship also exists in the animal kingdom. Extensive knowledge on the structure and function of quorum-sensing molecules has been obtained in less than a decade, and more autoinducers will surely be discovered in the coming years. A better understanding of interpopulation signaling between coexisting microbial populations would be of great interest as well. Since plants, animals, and microorganisms coexist in nature, it is not surprising that they have evolved to sense each other's presence. Fundamental research in quorum sensing will undoubtedly provide more precise insights into the mechanism(s) by which the expression of quorum-sensing-regulated genes is activated or inhibited. Such research will make it possible, for example, to conduct a more focused search for antagonists. The ability to generate bacterial quorum-sensing signaling molecules in transgenic plants offers the opportunity for disease control and for manipulating plant–microbe interactions to obtain improved crop production. Moreover, degradation of AHLs has not only been a preventive but also a curative biocontrol activity.

Using techniques that disrupt the quorum-sensing systems of pathogenic bacteria is a promising alternative to antibiotics in fighting bacterial infections. This new approach might also have value in aquaculture since a link between quorum sensing and virulence factor expression in several aquatic pathogens has been demonstrated.

The past decade has seen the emergence of a new way of thinking about bacteria. Rather than existing as individual cells in the environment (and in biologists' culture flasks, for that matter), bacteria grow in communities (notably biofilms) dominated by diversity, hence requiring forms of intra- and interspecies communication. Given that the vast majority of bacteria from soil and deep oceans are not even culturable, it will remain a major challenge to understand the bacterial world literally all around us. Efforts are ongoing to mine the genomes of the bacterial world for unusual and interesting natural products, which have yielded and will continue to yield new avenues of therapy against human infections and other diseases. It has not yet been proven that understanding bacterial communication will directly lead to new therapies against bacterial infections; however, studying the modes of chemical communication that exist in the bacterial world will surely enhance our understanding of diversity and the importance of quorum-sensing-based regulation of bacterial traits under different conditions and will provide new information on crop protection, human health, and for solving environmental problems.

Acknowledgement We are grateful to Prof. Robert JC McLean, Texas State University, USA, for his critical suggestions in the preparation of this manuscript. We would also like to acknowledge University Grants Commission, New Delhi for financial assistance in the form of a major research project.

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Chapter 7
Horizontal Gene Transfer Between Bacteria Under Natural Conditions

Elisabeth Grohmann

Abstract Conjugative plasmid transfer is the most important mechanism for bacteria to deliver and acquire genetic information to cope with rapidly changing environmental conditions. An update of knowledge of conjugative plasmid transfer in aquatic and terrestrial habitats, including environments of particular concern such as agricultural areas and contaminated soils and sediments, is presented. Environmental factors affecting horizontal gene transfer in nature are discussed. Recent advances in the design of in situ monitoring tools to assess conjugative plasmid transfer in nature and laboratory model systems to simulate environmental conditions are critically reviewed. The impacts of horizontal gene transfer on biodegradation as well as recent approaches to model conjugative plasmid transfer in complex microbial communities are presented.

7.1 Introduction

The "horizontal gene pool" refers to genetic information accessible to more than a single bacterial species, potentially resulting in phenotypes of one being acquired by another. This pool includes genes of mobile genetic elements (MGEs) and genes that are not mobile themselves, but may be mobilized by MGEs (Slater et al. 2008). Plasmids, bacteriophages, conjugative transposons, and integrative conjugative elements (ICE) are examples of MGEs. Plasmids as self-replicating MGEs generally provide accessory, but not essential functions to their hosts. In particular, traits that confer adaptations to locally restrictive conditions tend to be clustered on plasmids.

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_7,

In spite of the energetic burden imposed upon the host cell metabolism, plasmids can be considered as desirable elements for their host providing a mechanism for initiation of functions that are required for survival under environmental stress conditions but are dispensable in the absence of stress effectors (van Elsas et al. 2000).

Bacterial conjugation is one of the most important means of gene delivery enabling adaptation of bacteria to changing environmental conditions, including spread of antibiotic resistance genes, thereby generating multiple antibiotic-resistant bacteria.

As microorganisms occupy and adapt themselves to different ecological niches within the biosphere, their activities control global homoeostasis in large part. Through its effects on microbial adaptation, horizontal gene transfer (HGT) poses both challenges and opportunities for the control of global human and environmental health (Smets and Barkay 2005). The mobile gene pool or "mobilome" spans all kingdoms of life.

This chapter focuses on the bacterial mobilome, the gene pool available for transfer from one bacterium to the other and for acquisition by bacteria from other organisms in the same environment. The chapter is divided into sections dealing with HGT in different natural and anthropogenic habitats including soil, sediments, and aquatic environments Research performed by several groups on HGT modeling is summarized. Promising approaches to monitor HGT without cultivation of the cells as well as valuable approaches to assess HGT frequencies under natural conditions or conditions mimicking nature are discussed. The chapter ends with a description of technological prospects provided by transmissible traits encoded on MGE with respect to biodegradation and bioremediation in contaminated habitats, and perspectives for HGT research.

7.2 Horizontal Gene Transfer in Soil, Sediments, and Other Solid Surfaces

An excellent summary of the ecology of plasmid transfer was published by van Elsas et al. (2000). Van Elsas and coworkers issued key questions to be answered in the field of ecology of HGT. One of the most important questions is how efficiently do plasmids spread in the environment and how is this spread affected by environmental factors.

Van Elsas et al. (2000) defined a series of key abiotic and biotic factors that affect plasmid host fate in natural environments, presumably having a net effect on HGT. The stimulating abiotic factors include, among others, presence of nutrients, presence of colonizable surfaces, soil texture (e.g., high clay content favors HGT due to protection of plasmid hosts), physiological temperatures, presence of oxygen for aerobic microorganisms, etc. Biotic factors that enhance HGT include plant roots and other nutrient-rich colonizable surfaces, as well as soil animals

offering colonizable surfaces and interior environments, such as the gut of soil insects where plasmid transfer was demonstrated (Hoffmann et al. 1998, 1999). Many studies have reported the occurrence of conjugative transfer (mediated by plasmids or conjugative transposons) between bacteria in soil (e.g., Krasovsky and Stotzky 1987; Schofield et al. 1987; van Elsas et al. 1988a, b; Richaume et al. 1989; Henschke and Schmidt 1990; Smit et al. 1991, 1993; Pukall et al. 1996; Götz and Smalla 1996; van Elsas et al. 1998; van Elsas and Bailey 2002; Smalla et al. 2006; Ansari et al. 2008; Malik et al. 2008; Sobecky and Coombs 2009). These studies have conferred important knowledge on HGT in natural habitats; however, all possess natural restrictions or limitations. They focused on a specific habitat and/or a particular MGE or a particular class of MGEs, in most cases conjugative plasmids.

Van Elsas and Bailey (2002) reviewed the impact of different experimental approaches and the influence of key environmental factors on HGT in soil and the phytosphere. They demonstrated how structured bacterial communities such as biofilms and selective pressure affect HGT frequencies in natural bacterial consortia.

7.2.1 Environmental Factors Affecting HGT in Nature

Microbial growth in most natural environments is restricted by the quantity of nutrients present, which can dramatically limit population densities and activity (van Elsas and Bailey 2002). In soil, in particular, plant surfaces were shown to provide conditions for microbial colonization, mixing, and bacterial activity, resulting in locally enhanced densities of bacterial cells. These sites offer favorable conditions for gene exchange and have been named "hot spots" for HGT (van Elsas et al. 2000). Hot spots for HGT processes in soil include rhizosphere and belowground plant tissue (e.g., Pukall et al. 1996; Lilley and Bailey 1997; Kroer et al. 1998; van Elsas et al. 2000; van Elsas and Bailey 2002), the phyllosphere (e.g., Björklöf et al. 1995; van Elsas et al. 2000; Kay et al. 2002), manured soil (e.g., Götz and Smalla 1996; Heuer and Smalla 2007; Heuer et al. 2009) as well as guts of soil animals such as *Collembola* and earthworms (Daane et al. 1996; Hoffmann et al. 1998; Thimm et al. 2001).

7.2.2 Tools to Study Horizontal Gene Transfer in the Environment

Three different types of tools are applied to study HGT in nature:

1. *Direct disruptive tools*. These methods include extraction of bacteria from the environment and cultivation of the bacteria on selective media, followed by

molecular analysis. They have been applied to microcosms simulating plasmid transfer conditions in soil and the rhizosphere (e.g., Hoffmann et al. 1998; Kroer et al. 1998; Lilley et al. 2003) and to studies in the field, e.g., to investigate plasmid transfer in the phytosphere (e.g., Lilley et al. 1994; Lilley and Bailey 1997; van Elsas et al. 1998).

2. *Indirect tools*. These methods include plasmid DNA isolation, PCR on genes encoded by MGEs, such as antibiotic resistance genes and key transfer factors, and sequence analysis of MGEs or parts thereof. They have been applied to different terrestrial environments to detect sequences of MGEs supporting evidence of gene transfer potential. The indirect tools do not provide evidence of plasmid transfer, but they provide evidence of the presence of conjugative plasmids and of the respective transfer genes. Molecular detection of transfer genes and plasmid DNA isolation does not prove that gene transfer takes place or has taken place in the respective habitats. However, it supports evidence of gene transfer potential (e.g., Götz and Smalla 1996; Levin and Bergstrom 2000; Ochman et al. 2000; Smalla et al. 2000; Mendum et al. 2001; Ansari et al. 2008; Malik et al. 2008; Ansari 2009).
3. *Direct, nondisruptive tools*. These methods include fluorescence monitoring tools such as the use of plasmid donors with repressed *gfp*, coding for the Green Fluorescent Protein (e.g., Sørensen et al. 2003). These tools have been applied in particular to detect plasmid transfer events in biofilms (e.g., Christensen et al. 1996, 1998; Heydorn et al. 2000). Recent developments on nondestructive techniques to quantify plasmid transfer are summarized in the section "Monitoring HGT and assessing transfer frequencies".

7.3 Plasmid-Mediated Gene Mobilization in Soil

Plasmids drive HGT in soil; however, information on the diversity of plasmids and other MGEs in soil and the phytosphere is still scarce. Depending on plasmid isolation protocol, plasmids with different characteristics with respect to Inc group, host range, antibiotic and heavy-metal resistance, and conjugative and mobilizable abilities can be obtained. The most effective methods to obtain conjugative plasmids with plasmid mobilization capacity are bi- and triparental exogenous isolation. Conjugative plasmids are captured directly from environmental samples into recipient strains grown under selective laboratory conditions (Bale et al. 1988b; van Elsas and Bailey 2002). These tools have been successfully applied to soil and phytosphere habitats (e.g., Lilley et al. 1994; van Elsas et al. 1998; Malik et al. 2008). pIPO2 was shown to self-transfer and mobilize IncQ plasmids to various Gram-negative bacteria in the wheat rhizosphere under field conditions (van Elsas et al. 1998). Mercury-resistance plasmids that were able to mobilize IncQ plasmids such as RSF1010 were also found (van Elsas et al. 2000). Prevalence of these plasmids seemed to be enhanced under conditions of mercury stress.

7.3.1 Horizontal Gene Transfer in Metal- and Radionuclide-Contaminated Soils and Sediments

The presence of conjugative plasmids and antibiotic-resistance genes in anthropogenic soils from India and Germany by antibiotic resistance gene and key transfer factor-specific PCR and Southern hybridization was investigated in our laboratory. The abundance of resistance factors and broad-host-range conjugative plasmids in an urban park and an abandoned sewage field in Germany were compared with those from four different Indian sites, three agricultural fields with distinct irrigation history (irrigation with industrial wastewater from tannery or steel industries), and one agricultural field irrigated with groundwater (Malik et al. 2008). Samples from the abandoned sewage field and all the Indian soils demonstrated the occurrence of IncP-specific plasmid sequences like $oriT_{\text{IncP}}$ and the replication gene *trfA*, whereas in soil samples from the urban park, no IncP sequences were detected. Biparental exogenous plasmid isolation with bacteria detached from contaminated soils showed prevalence of conjugative IncPβ plasmids in the strongly polluted German site (abandoned sewage field) and the Indian agricultural field which had received wastewater from steel industries for many years. A similar conclusion was obtained by studies on multiple antibiotic- and heavy-metal-resistant bacterial isolates from highly heavy-metal-contaminated Indian soils for the presence of conjugative plasmids from Gram-negative bacteria. The presence of conjugative/mobilizable IncP plasmids in the isolates indicated their gene-mobilizing capacity with implications for potential dissemination of introduced recombinant DNA (Ansari et al. 2008).

Smalla et al. (2006) detected increased abundance of IncP-1β plasmids and mercury-resistance genes in mercury-polluted river sediments. They investigated river sediment samples from two mercury-polluted and two nonpolluted or less-polluted areas of a river in Kazakhstan for the presence of mercury-resistance genes and broad-host-range plasmids by PCR. An increase of the degree of mercury pollution corresponded to an increased abundance of mercury-resistance genes and of IncP-1β replicon-specific sequences detected in total community DNA (Smalla et al. 2006). Three different IncP-1β plasmids (pTP6, pTP7, and pTP8) were captured from contaminated sediment by the triparental exogenous plasmid isolation method. The plasmids conferred mercury resistance to their host, and the presence of a mercury-resistance transposon on these plasmids was demonstrated by hybridization. The nucleotide sequence of pTP6 revealed a backbone almost identical to that of the classical IncP-1β plasmid R751 (Smalla et al. 2006). This study provided further evidence of the role of IncP-1β plasmids in mediating maintenance and spread of adaptive traits such as mercury resistance, in bacterial communities.

Sobecky and Coombs (2009) summarized the state of the art of HGT in metal- and radionuclide-contaminated soils. Metal and radionuclide contamination in soils and in the subsurface poses a serious challenge to bacterial growth and survival because these contaminants cannot be transformed or biodegraded into nontoxic forms as it often occurs with organic xenobiotics (Sobecky and Coombs 2009). HGT

has played a major role in the dissemination of metal-resistance determinants among microbial communities (Sobecky and Coombs 2009). Metal-resistance genes were first detected on plasmids from diverse bacteria (Summers and Silver 1972; Silver et al. 1981). Subsequently, it was shown that transposons promote the acquisition of these plasmid-encoded metal-resistance genes (Liebert et al. 1999). Mercury-resistance operons often encode concomitant antibiotic resistance genes (Liebert et al. 1999; Gilmour et al. 2004). Baker-Austin et al. (2006) and Wright et al. (2006) demonstrated that heavy-metal contamination due to anthropogenic sources contributes to the dissemination of antibiotic-resistance genes by either coselecting for antibiotic-resistant bacteria carrying metal-resistance genes, located on the same MGE, or by selecting for cross-resistance encoded by multidrug efflux pumps exporting metals and antibiotics (Baker-Austin et al. 2006; Wright et al. 2006).

One of the best characterized metal-resistance loci is the *mer* operon, consisting of up to seven genes required for transport, catalysis, and regulation of mercury resistance (Barkay et al. 2003). Exogenous plasmid isolation was applied to isolate conjugative mercury-resistance plasmids from bacterial soil populations (Sobecky and Coombs 2009). Five different novel Hg^R plasmid groups were detected in the rhizosphere and phyllosphere of sugar beets (Lilley et al. 1996). A study on the soil bacterial populations associated with wheat roots showed that in soils amended with mercury, novel plasmid groups conferring Hg^R were recovered by exogenous plasmid isolation (Smit et al. 1998). This phenomenon emphasizes the endemic nature of MGEs conferring Hg^R resistance among soil microbial communities.

Three mechanisms that promote microbial heavy-metal resistance or tolerance are known: (1) metal reduction, (2) metal complexation, and (3) ATP-dependent metal efflux (Sobecky and Coombs 2009). The P_{IB}-type of prokaryotic heavy-metal-translocating ATPases detoxifies the bacteria by exporting Cd(II), Co(II), Pb(II), Ni(II), and Zn(II) (Sobecky and Coombs 2009). P_{IB}-type ATPase genes have been detected on MGEs from Gram-positive (Nucifora et al. 1989; O'Sullivan et al. 2001) and Gram-negative bacteria (Mergeay et al. 2003). Dissemination of horizontally acquired P_{IB}-type ATPase genes was shown by Sobecky and Coombs (2009).

Arsenic (As) occurs in four different oxidation states, As^{+5}, As^{+3}, As^{0}, and As^{-3}. It is a micronutrient used by a variety of microorganisms for cell growth and metabolism (Sobecky and Coombs 2009). Prokaryotic metabolic activity has been shown to be important in the transformations and subsequent mobilization/immobilization of As compounds (Stolz et al. 2006). The *ars* operon encodes a detoxification pathway for As, which can be chromosomally or plasmid-encoded (Sobecky and Coombs 2009). The operon contains numerous genes including *arsC* encoding arsenate reductase, which reduces arsenate to arsenite (Stolz et al. 2006). Phylogenetic analysis of more than 400 *arsC* sequences supported the role of HGT in the evolution and dissemination of arsenate reductase (Jackson and Dugas 2003).

Radionuclides cause severe environmental contamination problems for several reasons: (1) many radionuclides are heavy metals, and exposure to cells results in toxicity effects in addition to damage caused by radioactive decay; (2) radionuclides cannot be broken down or detoxified by transformation; (3) radionuclides are often present together with other environmental contaminants. This means that any

surviving organism in affected environments must be multiple-contaminant resistant; and (4) bacteria have only limited resistance mechanisms for radionuclides (Sobecky and Coombs 2009).

Dissimilatory metal-reducing bacteria such as *Geobacter sulfurreducens* (e.g., Lovley et al. 1991; Lloyd et al. 2000) and sulfate-reducing bacteria such as *Desulfovibrio desulfuricans* (Lloyd et al. 1999) contain electron shuttle systems that immobilize radionuclides by reduction to their less mobile forms.

Reduction of radionuclides and heavy metals such as Cr(VI) is carried out by two possible mechanisms. Indirect reduction could take place when Fe(II), Mn(II), and H_2S are generated by microbes during anaerobic respiration. The oxidation of these compounds to Fe(III), Mn(IV), and SO_4^{-2} could work to reduce metals such as U(VI) and Tc(VII). Indirect reduction has not yet been shown in situ, however. The alternative mechanism is direct enzymatic reduction, a process not fully understood (Sobecky and Coombs 2009). However, it is known that c-type chromosomes play an important role in dissimilatory metal-reducing bacteria (e.g., Shelobolina et al. 2007; Marshall et al. 2008) and in sulfate-reducing bacteria (Lovley et al. 1993; Payne et al. 2004). There is no direct evidence of HGT of genes required for enzymatic reduction; however, studies with c-type cytochromes demonstrated that HGT of these cytochromes can occur (Bertini et al. 2007; Sobecky and Coombs 2009). Analysis of 235 bacterial genomes revealed c-type cytochromes in nine cyanobacteria, *G. sulfurreducens*, and *Nitrosomonas europaea* (Sobecky and Coombs 2009).

7.3.2 Horizontal Gene Transfer in Mixed Waste Sites

Mixed waste in this section refers to anthropogenic contamination consisting of organic chemicals and radionuclides. Mixing of more than one contaminant at a waste site is important, as cocontaminants may interact with each other to enable or interfere with chemical transformation or contaminant transport. Variation in electron acceptors can result in the generation of different redox zones over small spatial scales (Barber et al. 1992; Cozzarelli and Weiss 2007; Sobecky and Coombs 2009).

Metal resistance and catabolic genes are often encoded on MGEs (for reviews see Liebert et al. 1999; Mergeay et al. 2003; Springael and Top 2004). Transposons that have been sequenced from environmental samples appear to encode only catabolic genes (for a review see Wyndham et al. 1994) or metal-resistance genes (e.g., Mindlin et al. 2001; Kholodii et al. 2002). A small number of plasmids contain genes for both (Sobecky and Coombs 2009). Of these, pJP4, pWW0, and pUO1 are self-transmissible plasmids (Kawasaki et al. 1981; Neilson et al. 1994; Pinedo and Smets 2005). Mobilization of pJP4 was demonstrated in soil (Neilson et al. 1994) and in bioreactors containing 2,4-D or 2,4-D and cadmium (Newby et al. 2000; Sobecky and Coombs 2009). It appears that exposure to toxic compounds such as 2,4-D and cadmium does not have a stimulating effect on the conjugative transfer of large catabolic plasmids (Sobecky and Coombs 2009).

Several cases of HGT among high and low G/C Gram-positive bacteria from mixed-waste have been reported. Most bacteria harbored large plasmids and could also tolerate toxic concentrations of U(IV) at low pH (Martinez et al. 2006). The frequency of HGT was higher among isolates from the contaminated site than from an uncontaminated site (Coombs and Barkay 2004).

7.3.3 Horizontal Gene Transfer in Agricultural Soils

Genomic approaches have revealed a large diversity of MGEs in soil and plant-associated bacteria, including plasmids, prophages, pathogenicity islands, and integrons. Pathogenicity islands are MGEs that account for rapid changes in virulence potential. They are known to have contributed to genome evolution by HGT in many bacterial pathogens (Dobrindt et al. 2004). Integrons are assembly platforms – DNA elements that acquire open reading frames embedded in exogenous gene cassettes – and convert them to functional genes by ensuring their correct expression (Mazel 2006; Heuer and Smalla 2007). Approximately 18% of bacterial isolates from the phytosphere of sugar beets were shown to harbor plasmids (Powell et al. 1993). Many were able to mobilize nonself transmissible IncQ plasmids (Kobayashi and Bailey 1994).

The exogenous isolation of MGEs was applied to capture MGEs from soil and phytosphere microbial communities (Smalla and Sobecky 2002). Antibiotic resistance or mercury resistance was often used as selective markers to exogenously isolate conjugative plasmids from the phytosphere of different crops (e.g., Lilley et al. 1996; Lilley and Bailey 1997; Smit et al. 1998; Schneiker et al. 2001; Malik et al. 2008) and from mercury-polluted soils (Dronen et al. 1998) in Gram-negative plasmid recipients. Biodegradative genes encoded on MGEs were captured from soils treated with 2,4-D, but not from untreated controls (Top et al. 1995, 1996). Two different cultivation-independent approaches were used to isolate naphthalene-catabolic genes from oil-contaminated soil in Japan (Ono et al. 2007). One approach was the construction of a broad-host-range cosmid metagenomic library; the other involved exogenous plasmid isolation. A cosmid clone was obtained that carried a naphthalene-catabolic pathway operon for conversion of naphthalene to salicylate. The operon was similar to the corresponding operon on the IncP-9 naphthalene-catabolic plasmid pDTG1. Using the exogenous approach the microbial soil community was mated with a *Pseudomonas putida* recipient. Transconjugants had acquired either a 200- or 80-kb plasmid containing all the naphthalene-catabolic genes for complete degradation of naphthalene. Both plasmids belong to the IncP-9 incompatibility group, and the naphthalene-catabolic genes are highly similar to those of other IncP-9 plasmids, namely, pDTG1 and pSLX928-6 (Ono et al. 2007).

Miyazaki and coworkers determined the nucleotide sequence of the exogenously isolated plasmid pLB1 involved in γ-hexachlorocyclohexane degradation (Miyazaki et al. 2006). pLB1 was isolated from hexachlorocyclohexane-contaminated soil and

transferred from *Sphingobium japonicum* to other alpha-proteobacterial strains by conjugative transfer. Thus, pLB1 may contribute to the dissemination of genes for γ-hexachlorocyclohexane degradation in agricultural soils (Miyazaki et al. 2006).

Conjugative plasmids encoding multiple antibiotic resistance were captured from animal manure used for soil fertilization (Smalla et al. 2000; Heuer et al. 2002, 2008; van Overbeck et al. 2002; Heuer and Smalla 2007; Binh et al. 2007, 2008). van Elsas et al. (1998) isolated mobilizing plasmids from the rhizosphere of wheat plants by using microbial communities detached from the rhizosphere as donors in triparental matings. Plasmid pIPO2 was isolated in *Ralstonia eutropha* on the basis of its mobilizing capacity. Replicon typing and plasmid sequencing showed that this 45-kb cryptic plasmid was not related to any of the known broad-host-range plasmids except plasmid pSB102 (Schneiker et al. 2001). Sequencing of plant-associated bacteria has revealed that many phytopathogenic and symbiotic bacteria harbor plasmids (Vivian et al. 2001; Zhao et al. 2005; Sundin 2007; Crossman et al. 2008; Li et al. 2008; Ding and Hynes 2009), pathogenicity or symbiosis islands (Arnold et al. 2003; Ramsay et al. 2006; Büttner et al. 2007; Nandasena et al. 2007; Nakatsukasa et al. 2008), or integrons (Szczepanowski et al. 2004; Gillings et al. 2005).

Agersø et al. (2006) investigated the effect of tetracycline residues in pig manure slurry on tetracycline-resistant bacteria and the tetracycline resistance gene *tet*(M) in soil microcosms. Four different types of microcosms were established, supplemented with combinations of pig manure slurry and a tetracycline-resistant *Enterococcus faecalis* strain encoding the *tetM* resistance gene. The concentration of both tetracycline-resistant bacteria (total CFU) and tetracycline-resistant enterococci declined rapidly in all four types of microcosms. *tet*(M) was detected longer than tetracycline-resistant enterococci could be isolated. This result could be due to the presence of viable but not culturable (VBNC) bacteria encoding *tet*(M), HGT of *tet*(M) to indigenous soil bacteria, or presence of free DNA, e.g., attached to soil particles. The concentration of tetracycline was approximately stable throughout the study, but the antibiotic concentration had no effect on prevalence of tetracycline-resistant bacteria (Agersø et al. 2006). The tetracycline residues present in the microcosms originated from pig manure slurry resulting from therapeutic treatment of the pigs. Tetracycline concentrations were similar to the actual concentration in manured agricultural soil. At this concentration, tetracycline did not appear to select for tetracycline-resistant bacteria, but it is degraded slowly in soil and may accumulate over time if manure containing tetracyclines is regularly amended to the soil (Agersø et al. 2006). As *tet*(M) was detected much longer than the original *E. faecalis* host, the resistance genes might form an antibiotic resistance reservoir in soil (Agersø et al. 2006).

Toomey et al. (2009) studied the HGT of antibiotic-resistance genes (plasmid- and transposon-encoded) between wild-type dairy isolates of lactic acid bacteria using an alfalfa sprout model. The plant model provided an environment that appeared to promote high transfer frequencies between all lactic acid bacteria pairs tested. Transfer frequencies ranged from 4.7×10^{-4} to 3.9×10^{-1} transconjugants per recipient. Dairy cultures can act as a source of MGEs encoding antibiotic resistance that can

be spread with high frequency to other lactic acid bacteria in plant environments (Toomey et al. 2009).

7.4 Horizontal Gene Transfer in Aquatic Environments

As for soil and other natural environments, the frequency of conjugative plasmid transfer among bacteria in aquatic environments appears to be controlled by the characteristics of hot spots (van Elsas et al. 2000). Aquatic ecosystems can be divided into different habitats, (1) the free (bulk) water phase, (2) the colonizable suspended matter, (3) sediment or sewage, (4) stones and other surfaces carrying biofilms (termed the epilithon) (Hill et al. 1996), and (5) aquatic animals. Availability of nutrients as well as colonizable surfaces is important due to support of large densities of metabolically active bacteria (Hill et al. 1994; Muela et al. 1994; van Elsas et al. 2000). Suspended matter is a preferred site for bacterial growth, resulting in bacterial densities higher than that in bulk water. Sediments rich in organic material can support bacterial population densities approximately three orders of magnitude higher than those found in bulk water (Ashelford et al. 1997; van Elsas et al. 2000). Bacterial biofilm communities are found in the epilithon on stones in rivers or lakes (Lock et al. 1984) and in percolating filter beds, which are nutrient-rich environments that support high population densities of metabolically active bacteria (Gray 1992; van Elsas et al. 2000). Hence, plasmid transfer frequencies in aquatic environments seem to depend mainly on the possibilities for the formation of mixed donor–recipient colonies or biofilms (van Elsas et al. 2000). In natural environments, competing, grazing, or antagonistic microflora can impart a significant effect on HGT rates, as donor and recipient cell numbers and physiological activities can be severely affected. Bale et al. (1987, 1988a, b) and Hill et al. (1994) investigated the transfer of epilithon-derived plasmids between different *Pseudomonas* isolates. Due to antagonistic effects, transfer frequencies on sterile stones in broth were higher than those on epilithon-covered stones in river water.

7.4.1 Evidence of Plasmid Transfer in Aquatic Environments

Most HGT studies in aquatic environments have been performed in microcosms, as they provide the advantage of controllable study conditions (van Elsas et al. 2000). HGT frequencies in microcosms are often revealed to be consistent with those obtained in situ (Ashelford et al. 1995, 1997). Microcosms such as flasks, sediment columns, activated sludge units, sewage filter beds, or small chemostats are constituted of enclosed samples of the environment they mimic or of synthetic approximations of environmental samples. Indigenous microorganisms and other factors that provide complexity to the system, in particular colonizable surfaces and/or nutrient sources, may be present, and temperatures may be controlled or

manipulated. Although these microcosms are still different from the environment they represent, they are valuable, as they are simple, reproducible, flexible, and offer the possibility to control and/or adjust individual parameters (van Elsas et al. 2000). In microcosms and in some in situ experiments, plasmid transfer between different bacteria was demonstrated in drinking water (Sandt and Herson 1991), river water and epilithon (e.g., Bale et al. 1987; Hill et al. 1994; Muela et al. 1994; Shintani et al. 2008), lake water (O'Morchoe et al. 1988; Jones et al. 1991; Popova et al. 2005), seawater (e.g., Goodman et al. 1993; Barkay et al. 1995; Dahlberg et al. 1998; Sobecky and Hazen 2009), marine sediment (e.g., Breittmayer and Gauthier 1990; Sandaa 1993; Rasmussen and Sørensen 1998), and sewage and wastewater (e.g., Gealt et al. 1985; Lebaron et al. 1994; Ohlsen et al. 2003). Thus, plasmid transfer seems to be part of the natural lifestyle of bacterial cells inhabiting these environments (van Elsas et al. 2000).

7.4.2 Evidence of Plasmid Transfer in Sewage Filter Beds and Activated Sludge Units

Sewage filter beds and activated sludge units represent aquatic environments of extreme nutrient availability, microbial mixing, and competition. Therefore, plasmid transfer should occur at maximum rates in these systems. Sophisticated microcosms have been designed to mimic natural conditions of sewage filter beds and activated sludge units (van Elsas et al. 2000). Plasmid transfer in percolating filter beds was first studied by Ashelford et al. (1995). Plasmid transfer between different *P. putida* strains in the filter biofilm was observed (Ashelford et al. 1995, 1997).

Plasmid transfer studies were also performed in laboratory-scale activated sludge units by Mancini et al. (1987) and McClure et al. (1989). Mancini and coworkers studied conjugative plasmid transfer between laboratory *E. coli* K12 strains and between wastewater-isolated *E. coli* strains. Transconjugants were detected throughout the microcosm, with highest frequencies, 2.5×10^{-3} transconjugants per donor for laboratory strains, in the settled sludge (van Elsas et al. 2000). McClure et al. (1989) investigated the fate of a *P. putida* strain harboring the mobilizable plasmid pD10 in activated sludge units. They demonstrated pD10 mobilization to indigenous sludge bacteria. This demonstrates that mobilization of nonconjugative plasmids can readily occur in nutrient-rich environments.

7.5 Modeling of Conjugative Plasmid Transfer

Several attempts to model conjugative plasmid transfer and conjugative mobilization using different mathematical models with distinct simplifications on the complex process of plasmid exchange between two organisms have been reported. Some recent models are described briefly. Gregory et al. (2008a) used COSMIC-rules,

an individual-based model for bacterial adaptation and evolution to study virtual transmission of plasmids within bacterial populations. Their simulations showed the spread of resistance (R) plasmids, compatible and incompatible, by conjugative transfer. Three case studies were examined: transfer of an R plasmid within an antibiotic-susceptible population, transfer of two incompatible R plasmids, and transfer of two compatible R plasmids. Rules for plasmid transfer, e.g., cost rules for plasmid maintenance versus benefit rules for plasmid maintenance were set up (Gregory et al. 2008a). Simulations were carried out for all the three case studies. Transfer of R plasmids was demonstrated to occur in the simulations of all the three case studies. The results support the original concept of the authors (Gregory et al. 2008b), e.g., incompatibility could be predicted by this model to be an important limiting factor for plasmid spread in bacterial populations.

The ability to simulate plasmid transfer has applications in studies of adaptive evolution, dissemination of antibiotic-resistant bacteria (DeNap et al. 2004), and the ability of microbial populations to degrade xenobiotics (Basta et al. 2004).

Inoue et al. (2009) investigated the occurrence and persistence of transconjugants that have acquired self-transmissible plasmids via conjugation by a simulation model on conjugative plasmid transfer in soil. Two conjugative plasmids with broad-host-range in Gram-negative bacteria, RP4 and pJP4, were applied to transfer studies in soil microcosms. The simulation model incorporated the survival dynamics of the donors, recipients, and transconjugants, and the conjugative plasmid transfer dynamics. Bacterial survival was modeled as a simple growth/decay process. Bacterial conjugation was described based on the mass action model (Levin et al. 1979). Transconjugants were assumed to show similar survival characteristics as recipients with positive/negative effects resulting from plasmid acquisition. They were assumed to act as secondary plasmid donors with transfer rates differing from those of the original donors (Inoue et al. 2009). The microcosm experiments demonstrated that transconjugants occurred in soil even if the concentration of the original plasmid donors declined rapidly. The introduced plasmid can persist in the microbial community if the indigenous transconjugants are excellent plasmid donors with a broad spectrum of plasmid hosts (De Gelder et al. 2005) and/or high transfer frequency (Newby et al. 2000). The study of Inoue et al. (2009) was the first to model bacterial conjugation in complex microbial populations.

Sudarshana and Knudsen (2006) attempted to model plasmid mobilization between *E. coli* donors and *Pseudomonas fluorescens* recipients on pea seeds and roots. They developed a mathematical model to predict mobilization rates and to estimate the proportion of triparental matings in which plasmid mobilization occurs. The simple mathematical model was based on the mass action model of Levin et al. (1979) that was also applied by Inoue et al. (2009). The model assumes that matings occur among uniformly distributed donor and recipient cells and cells grow at the same constant rate. Although these assumptions are not fully met in heterogeneous ecosystems such as soil, modifications of the mass action model have been successfully applied to predict bacterial conjugation in soil, the rhizosphere, and the phyllosphere (Knudsen et al. 1988; Richaume et al. 1989; Clewlow et al. 1990).

The study of Massoudieh and coworkers focused on exploratory modeling of HGT among surface-associated *E. coli* in the subsurface (Massoudieh et al. 2010). They developed a model and experimental system to quantify HGT in biofilms formed on granular porous media in microflow chambers. Important characteristics of this sophisticated model are mentioned briefly. To track the kinetics of the partners in the conjugation process, four main states of the bacteria in the conjugative plasmid transfer process were considered: (1) donors, (2) recipients, (3) transconjugants, and (4) donors in the exhausted state (donors which have to recover before reinitiating conjugative transfer). The simulations confirmed the strong dependence of the transfer rate on the concentration of donors and recipients and considered attachment and detachment rates of the bacteria involved. Studies on sandy media and on glass beads will verify the model and quantify the characteristics of each of the processes considered in the model (Massoudieh et al. 2010).

7.6 Monitoring Horizontal Gene Transfer and Assessing Transfer Frequencies

Several excellent papers have been published on visualization of HGT between bacteria in vitro. Most of them apply fluorescent tools; e.g., Babic et al. (2008) presented an elegant study on direct visualization of HGT between single cells of *E. coli* in real time using the fluorescent fusion protein SeqA–YFP. However, monitoring plasmid transfer and assessing HGT frequencies in complex microbial communities in situ remain a challenge. Sørensen et al. (2005) published a critical review on HGT studies in situ. Direct evidence of the extent of in situ plasmid transfer in natural environments has been obtained by identification of plasmid-encoded phenotypes, such as antibiotic resistance or heavy-metal resistance, following the introduction of donor strains. This approach relies on the cointroduction of a marked recipient strain or the emergence of identifiable phenotypes among the indigenous bacterial populations (Sørensen et al. 2005). Plasmid transfer frequency in bulk environments such as bulk water and bulk soil is low (transconjugant/donor typically $<10^{-5}$). In many cases, transfer could only be detected after nutrient enrichment (Sørensen and Jensen 1998). This is in contrast to hot spots of bacterial metabolic activity and HGT, such as the rhizosphere and phylloplane of plants and other culturable surfaces, where transconjugant/donor ratios can be as high as 10^{-3} or even 10^{-1} for indigenous as well as introduced plasmids (Lilley et al. 1994; van Elsas and Bailey 2002).

Modern approaches to detect and quantify plasmid transfer use reporter gene technology. Due to simple detection by fluorescence microscopy, only fluorescence reporter genes have been used for in situ monitoring of HGT in natural environments. Biofilms are uniquely suited for HGT due to high bacterial density and metabolic activity even in the harshest environments (Wuertz 2002). Insights into the extent of HGT in biofilms were obtained from approaches combining fluorescently labeled plasmids and bacterial strains with confocal laser scanning microscopy and quantitative image analysis (Haagensen et al. 2002; Molin and Tolker-Nielsen 2003).

Christensen et al. (1998) investigated transfer of the TOL plasmid in flow-chamber biofilms of *P. putida*. Transconjugants were preferentially found on top of recipient microcolonies. Invasive transfer from new transconjugants to recipients in the microcolony was not observed. Online monitoring of transconjugant proliferation showed that the plasmid was primarily transferred vertically following a small number of HGT events (Sørensen et al. 2005).

The spatial structure of the biofilm has a decisive role in HGT. Bacterial conjugation was analyzed by observing the physical environment encountered by donor cells migrating into a biofilm matrix (Wuertz et al. 2004). Transconjugants were found deep inside biofilms grown in flow cells, indicating the ability of donor cells to penetrate beyond superficial surface layers (Sørensen et al. 2005).

Aspray et al. (2005) investigated conjugative plasmid transfer in a soil-based microbial biofilm in flow cells amended with 2,4-D. A 2,4-D-degrading donor strain, *P. putida* harboring a 2,4-D-catabolic conjugative plasmid tagged with *gfp* (pJP4::*gfp*) was inoculated into the flow-cell chambers containing 2-day-old biofilm communities. Transfer of pJP4::*gfp* from the donor to the bacterial community was detected by green fluorescence as monitored by confocal scanning laser microscopy (GFP fluorescence was repressed in the donor due to the presence of a chromosomally encoded *lacI*^{q} repressor gene). A 2,4-D-degrading transconjugant was isolated from the flow-cell chamber belonging to the genus *Burkholderia* (Aspray et al. 2005).

Conjugative plasmid transfer and plasmid mobilization in multispecies biofilms in continuously operated small-scale biofilm reactors have also been analyzed in the author's group. The biofilm communities consisted of different Gram-positive bacteria belonging to the genera *Staphylococcus* and *Enterococcus*. Plasmid transfer was monitored by GFP fluorescence. Donors and transconjugants were distinguished by an additional nontransferable fluorescence label in the donor cells. Transconjugants were obtained in intrageneric and intergeneric matings. Transfer rates were in the range of 10^{-8} transconjugants/recipient (Schiwon, K., Arends, K., and Grohmann, E., personal communication).

The use of GFP-tagged reporter plasmids for in situ studies has certain limitations, however. The fluorescence of GFP can be affected by environmental conditions such as high salt concentrations, low pH, and lack of oxygen, which is particularly relevant in dense biofilms. Expression of GFP in metabolically inactive or weakly active cells can be weak or even absent. Thus, expression cannot easily be distinguished from background fluorescence (Sørensen et al. 2005). Further efforts are required to develop more robust fluorescence labels with higher expression levels under environmental conditions.

7.7 Spread of Biodegradation Traits

Springael and Top (2004) published an excellent article on the state of the art of HGT in connection with microbial adaptation to xenobiotics. Characterization of bacteria that degrade organic xenobiotics has demonstrated that they can adapt to

these compounds by the expression of novel catabolic pathways. Some appear to have evolved by assembly of horizontally transferred genes, followed by mutations and gene rearrangements. New types of xenobiotic catabolic MGEs have been detected recently, the so-called catabolic genomic islands, which integrate into the chromosome after transfer (e.g., Toussaint et al. 2003; van der Meer and Sentchilo 2003). The presence of such xenobiotic-degrading bacteria in the biosphere has important environmental applications, such as the cleanup of polluted sites, fate of pollutants in the ecosystem, and their ecotoxicology (Head and Bailey 2003).

Van der Meer et al. (1998) provided a case of a potential in situ catabolic pathway assembly. They isolated chlorobenzene-mineralizing bacteria from chlorobenzene-contaminated groundwater. The isolates degraded chlorobenzene by a well-described two-step process (van der Meer et al. 1998). Springael and Top (2004) postulated that catabolic "precursor" genes might be present in the bacterial community before its exposure to contamination or they might be provided by "migrating" bacteria. Springael et al. (2002) showed that HGT resulting in the acquisition of xenobiotic degradation genes appears to occur with high frequency and over a relatively short period of time. Sentchilo et al. (2003) investigated the 105-kb genomic island of a *Pseudomonas* species carrying the *clcRABD* gene cluster that encodes the mineralization of chlorocatechols by a modified ortho-cleavage pathway. Induction of HGT of the *clc* element by 3-chlorobenzoate was demonstrated. Sentchilo et al. (2003) were the first to show that a pollutant can regulate transfer of the MGE encoding its metabolism.

Bathe et al. (2004) analyzed the possibility of enhancing degradation of 2,4-D in a sequencing batch biofilm reactor with the conjugative plasmid pJP4 encoding genes for 2,4-D degradation. Transconjugants were detected both by culture and culture-independent approaches in the 2,4-D-degrading biofilm. A 90% 2,4-D degradation was observed in the bioaugmented reactor within 40 h, whereas a control reactor without the plasmid contained 60% of the initial 2,4-D concentration after 90 h. This study showed the increase of 2,4-D degradation by conjugative transfer of pJP4 from an introduced donor strain to the bacterial community of a laboratory wastewater treatment system and demonstrated that adaptation of a microbial community to a xenobiotic compound can be accelerated by HGT of the respective catabolic genes (Bathe et al. 2004).

Bioaugmentation by HGT to mixed microbial populations in laboratory and pilot-scale sequencing batch biofilm reactors treating synthetic wastewater containing benzyl alcohol was analyzed by Venkata Mohan et al. (2009). A *P. putida* plasmid donor chromosomally labeled with the gene for the red fluorescent protein (RFP), harboring a GFP-tagged TOL plasmid that confers degradation of benzyl alcohol, was used. Survival of a bioaugmented strain, conjugative plasmid transfer, and increased degradation of benzyl alcohol were detected in the laboratory-scale reactor, but not in the pilot-scale reactor (Venkata Mohan et al. 2009).

7.8 Conclusions

Conjugative plasmid transfer is the most important means to disseminate resistance and catabolic genes among bacteria and to acquire them from other bacteria to cope with changes in the local environment. Many data have been collected in recent decades on the mechanism of HGT; protein key players have been identified and their enzymatic mechanisms elucidated. The three-dimensional structure of protein complexes required for horizontal plasmid spread has been solved for plasmids from Gram-negative bacteria, and detailed information on regulatory circuits involved in plasmid transfer of the sex-pheromone-responsive plasmids from Gram-positive enterococci has been obtained.

Ecology of HGT has kept pace with the advances in basic molecular and biochemical research. Research on in situ plasmid transfer has proceeded tremendously in recent decades, in particular due to the combined efforts of molecular biologists and microbial ecologists in the field. Experimental evidence for in situ plasmid transfer has been obtained for diverse aquatic and terrestrial habitats, biofilms on all kinds of surfaces, inner surfaces and organs of soil and water animals, habitats ranging from very oligotrophic to extremely nutrient-rich environments. Horizontal plasmid transfer appears to be a component of the natural lifestyle of all known bacteria.

This chapter summarizes the current knowledge of horizontal plasmid transfer in diverse environments with a focus on contaminated habitats, pointing out the contribution of microorganisms, in particular, of their mobilome (all transmissible or mobilizable genes), to the cleanup of polluted environments and the application of MGE in technical bioaugmentation processes to increase biodegradation of xenobiotic compounds.

7.9 Future Recommendations

Second-generation sequencing technologies have generated a large number of full-genome sequences including those of not only many pathogenic microorganisms but also numerous indigenous bacteria. Moreover, some research groups have focused on sequencing of whole plasmid genomes and genomes of other MGE of interest due to their prevalence in hospitals or in the environment. Determination of complete plasmid sequences from different origins with respect to host background and habitat will enable the comparison of numerous plasmid backbones and help decipher the evolution of mosaic structures of plasmid genomes, eventually leading to predictability of plasmid adaptation to environmental changes/challenges in the future.

Recent advances in fluorescence reporter technology (multiple labels, higher fluorescence intensities, fluorescence less affected by environmental conditions) and continuous improvements of microscopy techniques with three-dimensional resolution will facilitate the assessment of plasmid transfer efficiency in complex

environments. In conjunction with steadily improving and more realistic models of conjugative plasmid transfer and plasmid mobilization in complex microbial communities, they will help determine plasmid transfer frequencies in nature and presumably in the near future enable us to predict HGT events as responses to environmental stress.

Acknowledgements I sincerely thank Miquel Salgot for critical reading of the manuscript. I regret that not all valuable contributions of colleagues in the field of ecology of HGT are included due to space limitations.

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Chapter 8
Molecular Strategies: Detection of Foodborne Bacterial Pathogens

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Abstract Conventional methods of pathogen identification have often depended on the identification of disease symptoms, isolation, and culturing of the organisms, and identification by morphology and biochemical tests. The major limitations of these culture-based morphological approaches, however, are the reliance on the ability of the organism to be cultured, the time-consuming nature, and requirement of extensive taxonomic expertise. The use of molecular methods can circumvent many of these shortcomings. Accordingly, there have been significant developments in the area of molecular detection of bacterial pathogens in the last 3 decades. We report here a brief overview of the molecular detection methods applicable to microbes from food.

8.1 Introduction

Diseases caused by contaminated food constitute one of the most widespread public health problems and are an important cause of reduced economic productivity in both developed and developing countries (Anon 2005). Every year approximately 76 million foodborne illnesses are reported in United States of which 325,000 become hospitalized and approximately 5,000 die. The costs in terms of medical care and lost productivity are estimated at between $6.5 and $34.9 billion (Buzby and Roberts 1997; Mead et al. 1999). The number of people in Canada who contract foodborne illness is estimated as 2.2 million annually (Anon 2005).

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_8,

Generally, due to the presence of very low numbers (<100 CFU g^{-1}) in the midst of millions of other bacteria it is difficult to detect specific bacterial food pathogens. These microbes seem hidden among a background of indigenous microflora, and substances within the foods themselves may hinder detection. There is also the difficulty of demonstrating that the strains recovered from a food sample are, indeed, pathogenic to humans (Sockett 1991). Rapid and simple detection of pathogenic organisms facilitate precautionary measures to maintain healthy food (Feng 1992).

One of the major limitations to research in microbial communities, and consequently the detection of bacteria in the environment, is the inability to isolate and grow in culture the vast majority of bacteria. There continues to remain a discrepancy between cell numbers obtained from direct and viable counts to the numbers actually occurring in vivo (Keer and Birch 2003). Furthermore, some bacteria have been shown to be unculturable but retain their viability after exposure to the environment and have thus been termed "non-culturable but viable" (NCBV) (Oliver 2005). This phenomenon complicates both the detection and enumeration of key pathogenic organisms. A number of species are described as entering the VBNC state and include a large number of human pathogens, including *Campylobacter* spp., *Escherichia coli* (including EHEC strains), *Listeria monocytogenes*, *Salmonella* and *Shigella* spp. and *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Oliver 2005). The genera of *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium botulinum*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, *Shigella* spp., and *Yersinia enterocolitica* comprise primarily foodborne bacterial pathogens (Kumar et al. 2002).

The characterization and detection of foodborne pathogens continue to rely on conventional culturing techniques, which include homogenization, enrichment in nonselective and selective media followed by plating in differential agar media to isolate pure cultures. Finally, phenotypic and genotypic characterization takes 3–4 days to confirm the results. Biochemical and immunological methods for the detection require substantial amounts of pure culture, whereas DNA-based methods can be performed with mixed cultures or community DNA. The final detection stage requires gel electrophoresis after polymerase chain reaction (PCR) steps and further sequencing of the amplified product, thus increasing the time and complexity of detection (Prasad and Vidyarthi 2009).

Currently, diagnostic laboratories are adapting molecular methods for routine detection of pathogens. With advances in molecular biology and biosystematics, the techniques available have evolved significantly over the past decade. In addition to conventional PCR, other technologically advanced methodologies, such as second generation PCR (real-time PCR) and microarrays which allow unlimited multiplexing capability, have the potential to bring pathogen detection to a new and improved level of efficiency and reliability (Mumford et al. 2006).

The rapid methods employed for the identification of foodborne microorganisms are discussed below.

8.2 Molecular Typing Methods for the Detection of Bacterial Pathogens

Conventional methods of pathogen identification have often depended on the identification of disease symptoms isolation and culturing of organisms, and identification by morphology and biochemical tests. The major limitations of these culture-based morphological approaches are the reliance on the ability of the organism to be cultured, the time-consuming nature of the lab analyses, and the requirement of extensive taxonomic expertise. The use of molecular methods can circumvent many of these shortcomings. DNA-based technologies such as the PCR have revolutionized molecular diagnostics and microbiological investigations.

8.2.1 PCR-Based Detection Methods

The PCR is a technique for in vitro amplification of specific segments of DNA by using a pair of primers (Nguyen et al. 1994). A million-fold amplification of a particular region can often be realized, allowing, among numerous other uses, the detection of specific genes within samples. PCR can be used to amplify genes specific to taxonomic groups of bacteria and also to detect genes involved in the virulence of foodborne bacteria (Finlay and Falkow 1988; Bej et al. 1994).

In our laboratory, for the amplification of *hly* gene (234 bp) a PCR technique was standardized (unpublished data). The reaction mixture was optimized with master mix as follows: 2.5 µl of 10× PCR buffer (20 mM Tris–HCl, pH 8.0 at 25°C, 100 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT) 50% glycerol, 0.5% Tween 20, and 0.5% Nonidet-P40), 1.0 µl of dNTP mix (25 mM), 1.0 µl of both forward and reverse primers (15 pmol), 0.2 µl of Taq DNA polymerase enzyme (5 U/µl), and 2 µl of DNA as template. Nuclease-free water was added to make the final volume 25 µl. PCR tubes containing reaction mixture were centrifuged and placed in a thermocycler. Cycling conditions included an initial denaturation step at 95°C for 5 min followed by 40 subsequent cycles consisting of heat denaturation at 95°C for 30 s, primer annealing examined at 53°C, 54°C, and 55°C, respectively for 1 min, and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min to ensure synthesis of all strands. The PCR products were electrophoresed on 1.5% agarose gel which showed a clear band at 234 bp (Fig. 8.1)

Several variations of the standard PCR have recently appeared and have contributed to the development of more sensitive detection methods. These are discussed below.

8.2.1.1 Multiplex PCR and Real-Time PCR

Multiplex PCR (mPCR) and real-time PCR (rPCR) are proving to be the most popular methods for microbial identification. The mPCR allows several targets to

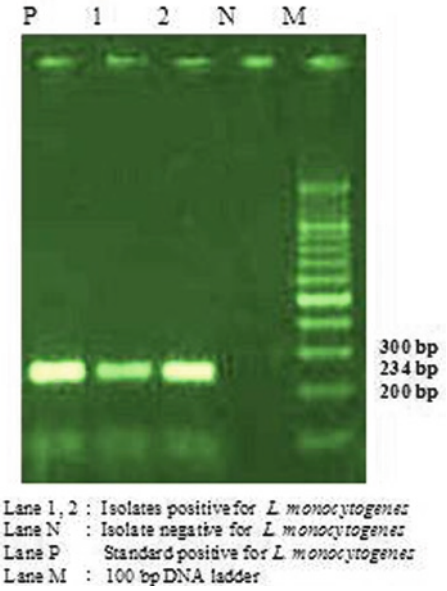

Fig. 8.1 *LL0* gene based detected of *L.* Monocytogenes using PCR

be co-amplified in one PCR by combining or "multiplexing" primer pairs (Newton and Graham 1997). Duffy et al. (2001) described PCR-based detection of foodborne pathogens including *L. monocytogenes*, *Salmonella* sp., *C.jejuni*, and *E. coli* O157:H7. A multiplex PCR protocol was reported for 13 species of foodborne pathogens (Cerniglia et al. 1997). Another protocol was reported by Park et al. (2006) for the simultaneous detection of *E. coli* O157:H7, *Salmonella* spp., *S. aureus*, and *L. monocytogenes* from kimchi (a Korean food preparation).

rPCR allows reactions to be characterized by the time amplification of the PCR product, which can be first detected by the use of a fluorogenic probe (Livak 2000). In recent years, there has been significant progress in the development of rPCR aimed at the quantitation of bacterial load in various food matrices. Its principle is based on the detection of a fluorescent signal, which is proportional to the number of amplicons in the tested sample (Higuchi et al. 1992, 1993; Lee et al. 1993; Livak et al. 1995). Nowadays, rPCR-based detection is frequently used for foodborne bacterial pathogen detection (Malorny et al. 2004; Poltronieri et al. 2009; Life Technologies 2010).

In recent years, PCR has become important as a technique for the detection and identification of bacteria. The main reason for its popularity is that DNA from a single bacterial cell can be amplified in about 1 h, which is significantly more rapid than times necessary for the methods described previously. However, the method can also amplify dead cells, and this makes data interpretation complex and is an issue that must be addressed, as it has long-term implications from legal perspectives. So care must be taken in designing experiments. Some investigators have detailed the PCR-based detection protocol for some foodborne bacterial pathogens (Islam et al. 1993; Keer and Birch 2003).

Diagnostic PCR has been greatly improved by the introduction of second-generation PCR, known as real-time PCR, where closed-tube fluorescence detection and quantification during PCR amplification (in real time) occurs, eliminating the need for laborious post-PCR sample processing steps which greatly reduces the risk of carryover contamination. Using real-time PCR it is possible not only to detect the presence or absence of the target pathogen, but also to quantify the amount present in the sample. Enumerating the pathogen upon detection is crucial for estimating the potential risks with respect to disease development and provides a useful basis for disease management decisions.

In another PCR assay targeting the 3′-prime end of the eae gene (*E. coli* attaching and effacing) of *E. coli* O157:H7 (no RT-PCR) was found to be specific, with sensitivity being 1 pg DNA or 10^3 CFU PCR per reaction (Uyttendaele et al. 1999). Furthermore, studies were carried out to determine the effect of the food matrix and sample preparation method on PCR detection of nonviable cells using heat-killed bacteria in ground beef. Sample preparation methods included centrifugation, buoyant density centrifugation (BDC), immunomagnetic separation (IMS), chelex extraction, and swabbing. It was found that IMS was the only method which did not produce false positive results, provided the number of cells was below 10^8 CFU g^{-1}. Above this number, IMS produced a false positive, which is a severe limitation of this approach.

8.2.1.2 Random Amplified Polymorphic DNA

The random amplified polymorphic DNA (RAPD) technique was first employed by Williams et al. (1990) to examine human DNA samples from anonymous individuals. Earlier, several authors reported on the application of the RAPD technique for the analysis of microbial DNA (Wagner et al. 1996; Byun et al. 2001). The method uses random primers (Williams et al. 1990) and can be applied to any species without requiring any information about the nucleotide sequence. The amplified products from this analysis exhibit polymorphism and thus can be used as genetic markers. The RAPD band, however, does not allow distinction between hetero- and homozygous states. The fragments are scored as dominant Mendelian elements, and the protocols are relatively simple.

Hamza et al. (2009) described a RAPD protocol for lactic acid bacteria identification from traditional Sudanese sour milk. The band pattern generated in the analysis represents genome characterization of a specific bacterial strain (Welsh and McClelland 1990). In addition, the method has the potential for analyzing phylogenetic relationships among closely related species (Williams et al. 1990) and can distinguish between strains within a species.

8.2.1.3 Restriction Fragment Length Polymorphism

The restriction fragment length polymorphism (RFLP) procedure involves isolation of DNA, digestion with restriction endonucleases, size fractionation of the resulting

DNA fragments by electrophoresis, DNA transfer from the electrophoresis gel matrix to a nylon membrane, preparation of radiolabeled and chemiluminescent probes, and hybridization to membrane-bound DNA (Olive and Bean 1999).

The probes can be labeled with detectable moieties, such as radioactive isotopes, enzyme-colorimetric, or enzyme chemiluminescent substrates (Arbeit 1995; Olive and Bean 1999). Due to the species and strain differences in the location of the restriction enzyme sites and with the specificity of the probe, the resulting fingerprint is simplified and therefore easier to analyze. The rRNA probe is more applicable for a wide variety of bacteria than other probes that are more species or strain-specific. The use of this probe for characterization is called ribotyping where restriction enzyme digestion and Southern blot hybridization are used together for analysis. Since the ribosomal operons in bacteria are organized into 16S, 23S, and 5S rRNA and are often separated by noncoding spacer DNA (Towner and Cockayne 1993), the probe can be either one of the rRNA genes or a mixture or parts of the rRNA genes and the spacer sequences. Hybridization patterns differ depending on the probe used (Saunders et al. 1990). Labeled probes containing *E. coli* 23S, 16S, and 5S rRNA sequences are most often used for ribotyping (Bingen et al. 1994).

Ribotyping has been shown to be advantageous in identifying strains, such as *Carnobacterium* species (*C. divergens*, *C. piscicola*, *C. gallinarum*, and *C. mobile*) which are difficult to type with classical phenotypic methods. Kabadjova et al. (2002) established a rapid PCR-RFLP-based identification scheme for four closely related *Carnobacterium* species (C. *divergens*, *C. piscicola*, *C. gallinarum*, and *C. mobile*) that are of interest to the food industry. Using the rapid PCR-RFLP scheme, three isolates previously incorrectly identified as *C. divergens* (INRA 508, INRA 586, and INRA 515) were reclassified as *C. piscicola*. Similarly, four isolates identified as *C. piscicola* (INRA 545, INRA 572, INRA 722, and ENSAIA 13) were reclassified as *C. divergens* based on the patterns obtained by the 16S–23S ISR-RFLP methods.

Manceau and Horvais (1997) used RFLP analysis of rRNA operons to assess phylogenetic diversity among strains of *Pseudomonas syringae pv. tomato*. They successfully established the close relationships existing between *P. syringae* and *P. viridiflava* species.

8.2.1.4 Amplified Fragment Length Polymorphism

Amplified fragment length polymorphism (AFLP) analysis was developed by a team led by Marc Zabeau at Keygene N.V., Wageningen, The Netherlands (Zabeau and Vos 1993; Vos et al. 1995). Vos et al. (1995) described the principle of the AFLP fingerprinting technique. AFLP is a variation of RAPD and is able to detect restriction site polymorphisms without prior sequence knowledge using PCR amplification for the detection of restriction fragments (Zabeau and Vos 1993; Vos et al. 1995; Blears et al. 1998; Mueller and Wolfenbarger 1999).

Basically, AFLP is a genome fingerprinting technique based on the PCR amplification of only certain fragments that have been the result of restriction digestion of the whole genome (Vos et al. 1995; Lin et al. 1996; Olive and Bean 1999). The basic procedure includes enzyme digestion by two restriction enzymes that yield DNA fragments with two different types of sticky ends. To these ends, adapters are ligated to form templates for the PCR. The selective amplification reaction is performed using two different primers containing the same sequence as the adapters, but extended to include one or more selec tive bases adjacent to the restriction site of the primer. Only fragments that are a complete match are amplified. This technique results in about 30–40 DNA fragments, some of which are species-specific while others are strain-specific (Janssen et al. 1996; Koeleman et al. 1998; Jackson et al. 1999; Jureen et al. 2004; Melles et al. 2007).

AFLP analysis is one of the most robust multiple-locus fingerprinting techniques among genetic marker techniques that have been evaluated for genotypic characterization (Koeleman et al. 1997). Restrepo et al. (1999) used AFLP to characterize the genetic relationships between *Xanthomonas axonopodis pv. Manihotis* strains. The study of Janssen et al. (1996) revealed extensive applicability of AFLP in bacterial taxonomy through comparison of newly obtained data with results previously obtained by well-established genotypic and chemotaxonomic methods such as DNA hybridization and cellular fatty acid analysis.

8.2.2 Pulsed-Field Gel Electrophoresis

Schwartz and Cantor (1984) described the pulsed-field gel electrophoresis (PFGE) method to produce a molecular karyotype from the chromosomal DNA of yeast *Saccharomyces cerevisiae*. PFGE is based on the digestion of chromosomal DNA by using rare cutting enzymes. The use of these enzymes minimizes the total amount of DNA fragments.

This method is capable of separating large DNA molecules (up to 2,000 kb) by applying alternately pulsed electric fields established perpendicular to each other and of which one is inhomogeneous. The basic principle of PFGE is the use of successive alternating electric fields which allow the DNA molecules to continuously change their direction of migration. The large DNA molecule will uncoil and elongate parallel to an electric field such that it can enter a pore opening in the agarose. When the electric field is turned off and a new electric field is applied perpendicular to the opened DNA, the molecule must re-orient itself to enter a new opening. The pulse time (ramping) and electron force (gradient) are constantly increased to achieve better separation of all sizes of DNA fragments (Towner and Cockayne 1993).

According to Arbeit (1995), PFGE is highly discriminatory and superior to many other microbial typing methods. The method is capable of differentiation between species and strains involved in foodborne outbreaks and therefore has been investigated for use in epidemiological studies such as with *Campylobacter*

coli, *C. jejuni* (Yan et al. 1991), *L. monocytogenes* (Brosch et al. 1991), and *S. aureus* (Schlichting et al. 1993).

In 1996, PFGE became the standard procedure for bacterial foodborne disease outbreak analysis (Swaminathan et al. 2001) due to its discriminatory capabilities (Gerner-Smidt et al. 2006). Uniform guidelines for performing PFGE and interpretation of the data have been established to confirm reproducibility among laboratories (Tenover et al. 1995). Therefore, PFGE is considered the "gold standard" for molecular-based studies. It has become the preferred subtyping method for networks that have been created within the United States (PulseNet) and Europe (PulseNet Europe) for surveillance and for collection of PFGE fingerprints of bacteria related to foodborne infections (Swaminathan et al. 2001; Rodríguez-Lázaro et al. 2007). Currently, PulseNet USA has standardized PFGE protocols for *Shiga toxigenic E. coli* O157, *S. enterica*, *Shigella* spp., *L. monocytogenes*, thermotolerant *Campylobacter* spp., and *V. cholerae* and *S. enterica sv. Braenderup strain H9812* digested with *Xba*I as the universal standard (Gerner-Smidt et al. 2006).

8.2.3 Biosensors

A biosensor is defined as a device or instrument comprising a biological sensing element coupled to a transducer. The biological sensing elements might include enzymes, organelles, antibodies, whole cells, DNA, and tissue. Transducers include electrochemical, calorimetric, optical, acoustical, or mechanical types (Richter 1993).

Microfabrication technology has enabled the development of electrochemical DNA biosensors with the capacity for sensitive and sequence-specific detection of nucleic acids. The ability of electrochemical sensors to directly identify nucleic acids in complex mixtures is a significant advantage over approaches such as PCR that require target purification and amplification. Application of DNA sensor technology to infectious diseases has the potential for recognition of pathogen-specific signature sequences in biological fluids (Liao et al. 2007).

Immobilization of a DNA probe on the desired substrate is the most crucial step in developing the electrochemical biosensor because sensitivity, specificity, and reproducibility are significantly affected by this step. For effective binding of DNA to its substrate, the terminus of the DNA or the surface of the substrate must be functionalized. Affinity binding of streptavidin and biotin has been successfully used for immobilization of DNA probes. Gold substrates are also gathering special attention due to their covalent attachment with thiolated DNA. This technology has a special interest in the search for rapid, portable, and low-cost testing systems. Electrochemical biosensors have been successfully used to detect *E. coli* 0157:H7 DNA combined with PCR (Berganza et al. 2007). A biosensor combined with gold nanoparticles (GNPs) has been used for the rapid detection of food pathogens (Leonard et al. 2003). Nanometer-sized gold particles have been used for the detection of specific DNA sequences (Daniel and Astruc 2004). As functionalized chemistry is not popular because of costs involved, an approach was proposed to use

nonfunctionalized GNP for the detection of dsDNA and ssDNA (Huixiang and Rothberg 2004; Sangchul et al. 2009). In this method, citrate-coated GNPs have a characteristic red color in the colloidal state. The aggregation of GNPs can be readily induced by the addition of salts resulting in a purple color. The difference in color is visualized with the unaided eye. The negatively charged GNP has an electrostatic interaction with ssDNA which can uncoil so that its hydrophilic negatively charged phosphate backbone is exposed to aqueous solution and DNA bases interact with the GNP surface by Vander Waals forces. These interactions add negative charge to GNPs and enhance their repulsion. Such properties have been exploited to design a biosensor which can detect a PCR product directly in the same tube within minutes.

Major improvements in signal intensity of a biosensor have been achieved, contributing significantly toward our goal of developing a microfluidics-based "lab-on-a-chip" electrochemical sensor assay for the detection of bacterial pathogens, including *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, and *Enterococcus faecalis* (Liao et al. 2007). An evanescent (i.e., quickly fading) wave fiber optic biosensor was used to detect the bacteria in 10 and 25 g ground beef samples. The biosensor uses a 635-nm laser diode to direct light onto optical fiber probes, which generates the evanescent wave. Fluorescent molecules within the evanescent field are excited and a portion of the emission recouples into the fiber probe. A photodiode detects and quantifies the fluorescent signal. A sandwich immunoassay was utilized, which allowed the detection of 9.0×10^3 CFU g^{-1} for 25 g samples and 5.2×10^2 CFU g^{-1} for the 10-g sample. No false positives were obtained with results obtained 25 min after sample processing (Demarco and Lim 2002).

8.2.4 Microarrays

The DNA microarray technology was originally designed to study gene expression and generate single nucleotide polymorphism (SNP) profiles. Currently, it serves as a diagnostic technology for emerging pathogens. Microarray technology offers a platform for unlimited multiplexing capability. Thousands of specific DNA or RNA sequences can be detected simultaneously on a small glass or silica slide measuring about 1–2 cm^2 (Aitman 2001) using microarray technology.

DNA microarrays consist of a solid surface (glass, silicon, nylon substrates) to which a large number of probes, DNA fragments, or oligonucleotides are immobilized that will hybridize to fluorescently labeled target DNA from the sample (Call 2005). The target can be genomic DNA isolated from the sample or an amplified PCR product. The DNA microarray is basically of two types, genomic microarrays and oligonucleotide arrays. In genomic DNA microarrays, the probes are complete genes or their fragments from a strain of a microorganism, while in oligonucleotide microarrays the target DNA hybridizes 18–70

nucleotide-length oligonucleotides. Although both types of microarrays can be used, pathogen detection, oligonucleotide microarrays are commonly chosen for the detection of either genomic DNA directly or the PCR-amplified portion of the genomic DNA, such as rRNA genes or virulence genes (Kostrzynska and Bachand 2006).

Microarrays have been developed for the identification of foodborne bacterial pathogens belonging to *Bacillus* spp., *C. jejuni*, *E. coli*, *L. monocytogenes*, *S. enterica*, *Shigella dysenteriae*, *Staphylococcus* spp., and *Vibrio* spp. (Call et al. 2003; Chiang et al. 2006; Garaizer et al. 2006; Sergeev et al. 2006; Eom et al. 2007) and for the discrimination of multiple pathogens and their virulence factors (Sergeev et al. 2006; Wang et al. 2007) in the case of food poisoning outbreaks and biological warfare (Sergeev et al. 2004; Wang et al. 2007).

In order to design a method for accurate detection and identification of foodborne pathogens, Kim et al. (2008) used comparative genomics to select 70 mer oligonucleotide probes specific for 11 major foodborne pathogens (ten overlapping probes per pathogen) for use in microarray analysis. Researchers analyzed the hybridization pattern of this constructed microarray with the Cy3-labeled genomic DNA of various foodborne pathogens and other bacteria. A highly specific hybridization pattern with the genomic DNA of each pathogen was observed. Microarray data were analyzed and clustered using the GenePix Pro 6.0 and GeneSpring GX 7.3.1 programs. The dendrogram revealed the discriminating power of the constructed microarray. Each foodborne pathogen clustered according to its hybridization specificity and nonpathogenic species were discriminated from pathogenic species. This method can be applied for rapid and accurate detection and identification of foodborne pathogens in the food industry. In addition, genome sequence comparison and DNA microarray analysis have a powerful application in epidemiologic and taxonomic studies as well as in the food safety and biodefence fields.

8.2.5 Integrated Systems

In the past few years some integrated systems (i.e., lab-on-a-chip) have grown, and some have been reported for the detection of bacterial pathogens (Kopp et al. 1998; Liao et al. 2007). These systems are popular because they decrease analysis times and increase efficiency of detection.

Recently, Lu et al. (2008) developed an on-chip immunoassay that detects an intracellular antigen of *L. monocytogenes* (Aad) based on polystyrene beads functionalized with the Aad antibody. Polystyrene beads were mixed thoroughly with cell lysate in the microfluidics channel so that beads were bound with the antigen in the lysate. The beads were exposed to fluorescently labeled Aad and the detected bacterial concentration was inversely proportional to the fluorescence intensity from the beads after washing. This chip can be useful for immunoassays based on cell lysates. Woolley et al. (1996) described the integration of PCR and capillary electrophoresis in a microfabricated DNA analysis device. The approach combines

thermal cycling with high-speed DNA separation by the CE chips. This system provided an assay of genomic Salmonella DNA in about 45 min. Andreas Manz's group has used a micromachined chemical amplifier to perform PCR in continuous flow at high speed (Kopp et al. 1998). The authors report that input and output of DNA are continuous, and amplification is independent of input concentration. They have reported that *Neisseria gonorrhoeae* was investigated and a 20-cycle PCR was completed in 90 s to 18.7 min, depending on flow rate.

An advanced nucleic acid analyzer (ANAA) was described by Lawrence Livermore National Laboratory for the detection of bacterial pathogens such as *Erwinia herbicola*, *Bacillus subtilis*, and *B. anthracis* (Belgrader et al. 1998). The instrument was composed of ten silicon reaction chambers with thin-film resistive heaters and solid-state optics. The authors reported that detection times were as short as 16 min and that 10^2–10^4 organisms per ml could be detected. The instrument allows for rapid analysis, low-power consumption, real-time monitoring, and for ruggedness due to lack of moving parts.

8.3 Conclusions and Future Prospectives

It was not the intent for this review article to list all the organisms that have been detected using molecular techniques but to show the range of new methods that are applicable for detecting bacteria in food samples. Foodborne pathogen identification is an important aspect of human health care. Isolation and identification of foodborne pathogens by biochemical and immunological methods are time-consuming and have less sensitivity compared with molecular methods. DNA polymorphism among the different species of bacteria has been exploited to identify food pathogens.

PCR methods have been developed for the identification of these bacterial pathogens. PCR is an effective, rapid, reliable, and sensitive technique for the detection of genes of bacterial pathogens from various foods (Park et al. 2006). The 5′ nuclease multiplex PCR assay has also found applications in simultaneous screening of bacterial pathogens in food commodities and various environmental samples. The method will be also effective for slow-growing or nonculturable microorganisms.

The electrophoresis-based methods described in this chapter (mPCR, RAPD, RFLP, AFLP, and PFGE) are time-consuming and laborious. RFLP requires pure culture for the discrimination of bacteria at the species level. The disadvantages of the RAPD technique are that standardization of concentration of primers and templates are needed to make reproducible amplification products, and most of the RAPD markers are dominant, i.e., it is difficult to distinguish between similar DNA sequences amplified. A problem related to AFLP analysis is the incomplete digestion of chromosomal DNA which may result in an aberrant AFLP pattern (Lukinmaa et al. 2004). PFGE has been considered the "gold standard" in identifying the causative organisms in cases of food poisoning, and water and hospital epidemics. PFGE has become the standard procedure for bacterial foodborne disease outbreak

analysis (Swaminathan et al. 2001) due to its discriminatory capabilities (Gerner-Smidt et al. 2006). Although the method is reliable and accurate, sample preparation and analysis are time-consuming, i.e., the method is slow.

Real-time PCR permits the acquisition of more rapid results with minimal manipulation. It is now possible to follow the amplification in real time, thus eliminating laborious postamplification processing steps such as gel electrophoresis. Real-time PCR offers better multiplexing possibilities; however, due to the availability of dyes emitting fluorescence at different wavelengths, multiplexing is still limited. Thus, detection of more than a few pathogens is currently not possible using these systems.

The microarray technology is currently a new and emerging pathogen diagnostic technology which, in theory, offers a platform for unlimited multiplexing capability. Tens of thousands of such probes can be spotted in a defined and addressable configuration on the glass slide forming the chip. The unlimited capability for simultaneous detection of pathogens offers much promise for microarrays to detect all relevant pathogens within a specific food matrix. In food microbiology, the development of microarrays for diagnostic applications is a recent development in this field, and has been detailed in this chapter. Microarrays have allowed for more rapid analyses; however, there are drawbacks to its use. Microarray instruments are expensive, of limited availability, and require specialized knowledge and training to extract useful information from the huge amount of data generated. This limits the broad application of microarray technology in ordinary laboratories. The effort to add a quantitative aspect to microarrays must continue and more work is needed to address the challenges of studying food samples where contaminants such as organic substances and heavy metals may interfere with DNA hybridization and affect the performance of microarrays.

Thus far, microbial biosensors and bioassays have been applied more for the detection of food additives and food contaminants than in direct monitoring of food pathogens (Table 8.1). Although biosensor research has sporadically appeared in the literature over 2 decades, few biosensors are commercially available. Major drawbacks include the delicate nature of the biological component and the miniaturization of the electrical components. As electronic innovation continues to deliver smaller and more reliable electronic devices and as the biological sciences continue to develop the unique understanding of enzyme and microbial genetics, the future will see reliable biosensors for the detection of biological events on-line. The food industry will significantly benefit from developments in rapid detection of microorganisms.

Although the above-described methods are highly specific and accurate, utmost care must be taken to standardize methods to isolate DNA from microbes in food samples. The DNA of dead microorganisms (VNBC) is also present which can amplify and give false positive results. Ethidium monoazide can be used to separate dead and viable bacteria (Rudi et al. 2002; Nogva et al. 2003; Keer and Birch 2003; Rudi et al. 2005). BDC also termed floatation, may be used successfully as a prior sample treatment to eliminate free DNA in samples (Wolffs et al. 2005). This can lower the risk of false positive results by avoiding DNA from VNBC bacteria.

Table 8.1 Summary of methods used to detect *Escherichia coli* O157:H7

Method	Approx. detection time	Detection limit	Selected references
Plating/culturing	1 day to 1 week	Low CFUs	Silk and Donnelly (1997)
Biochemical tests	1 day to several days	Low CFUs	Adams and Moss (1995)
ELISA	12 h to 2 days	10–100 CFU/ml	Gehring et al. (1999)
PCR	2–24 h depending on enrichment	10^2–10^5 CFU/ml	Uyttendaele et al. (1999)
Multiplex PCR	24 h	1–2 CFU/ml	Hu et al. (1999)
RT-PCR	6–12 h	10^7 CFU/ml	Yaron and Matthews (2002)
Laser-induced fluorescence	Few hours	Single organism	Johnson et al. (2001)
Fiber optic biosensor	ca. 30 min	5.2×10^2 CFU/g	Demarco and Lim (2002)
SPR biosensor	1 h	5×10^7 CFU/ml	Fratamico et al. (1997)
Microarrays	<1 h	55 CFU/ml	Call et al. (2001)
Integrated systems (lab-on-a-chip)	16–45 min	10^2–10^4 organisms/ml	Belgrader et al. (1998)

Due to inherent limitations in the methods developed thus far, it is unlikely that any one detection system will be suitable for monitoring genetically modified microorganisms. Similarly, the detection of recombinant microorganisms in the food microbiology industry may become an issue of interest that will stimulate further investigations into molecular methods for food microbiology.

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Chapter 9
Recent Advances in Bioremediation of Contaminated Soil and Water Using Microbial Surfactants

Achlesh Daverey and Kannan Pakshirajan

Abstract Environmental contamination by improper disposal of industrial, mining, agricultural, municipal, and other residues is known worldwide. Various chemical-, physical-, and biological-based methods are currently being developed for removal of such pollutants from soil and water. Among these techniques, biological treatment, or remediation using microbes, is one of the most promising techniques, mainly because of its cost-effectiveness and essentially complete destruction of numerous pollutants. The major requirement for this technique is survivability of the degrading microorganisms during the process. Biosurfactants, particularly microbial surfactants, play a vital role in cases where pollutants are not readily bioavailable, by increasing the apparent water solubility of the pollutants, which could be achieved either by ex situ addition or in situ production of biosurfactants by microbes. However, due to wide application potential of microbial surfactants in the environmental sector, it is important to know their mechanisms of action, recent advances in bioremediation processes, and other possible applications. The goal of this chapter is, therefore, to provide an overview of the different types of microbial surfactants and sources, their roles in several bioremediation processes, and recent advances in the field.

9.1 Introduction

Pollution of soil and water due to toxic heavy metals, polycyclic aromatic hydrocarbons (PAHs), petrochemicals, pesticides, and herbicides is increasing due to increases in global population, industrialization, and urbanization. These

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_9,

contaminants are generated as by-products of industries, mining, and agriculture and lead to soil and water contamination, if released into the environment without proper treatment. Accidental spillage may also lead to environmental problems. Due to potential toxicity, mutagenicity, and carcinogenicity of these contaminants, treatment of contaminated soil and water has become a major public health issue worldwide. Several conventional physical and chemical methods are available to treat contaminated sites; however, high cost and inability to destroy pollutants completely are their major limitations. The term "bioremediation" refers to remediation of contaminated soil and water by the use of microorganisms or their products (e.g., enzymes, microbial surfactants, etc.) and has shown excellent promise in terms of being cost-effective along with the potential to degrade many contaminants completely. The prerequisite for this method is a suitable microbial culture, which can survive in the contaminated media and degrade the contaminants efficiently and completely (Kosaric 2001). Bioremediation can be either in situ or ex situ, where the former is more appealing. In situ bioremediation often involves direct addition of microorganisms to the contaminated site; microorganisms utilize contaminants as the sole source of carbon or use them along with other carbon sources by cometabolism. Availability of contaminants that are highly water-insoluble is a limiting factor for microbial growth and biosurfactants, particularly microbial surfactants, play a vital role in bioremediation by increasing the apparent water solubility of the contaminants. Due to the wide application of microbial surfactants in the environmental sector, it is important to appreciate their mechanisms of action, recent advances in bioremediation processes, and other possible applications. This chapter provides an overview of the different types of microbial surfactants and sources, their role in several bioremediation processes, and recent advances in the field.

9.2 Microbial Surfactants/Biosurfactants

Microbial surfactants, commonly known as biosurfactants, are surface-active agents produced by a variety of microorganisms when grown on water miscible or oily substrates (Mukherjee et al. 2006). Similar to chemical surfactants, they possess a polar (hydrophilic) head and nonpolar (lipophilic) tail and are capable of forming micelles and reverse micelles as shown in Fig. 9.1. Also, microbial surfactants have the capability of reducing surface and interfacial tensions between the two phases.

9.2.1 Sources and Types of Biosurfactants

Biosurfactants are produced by bacteria, yeast, and fungi; therefore, they are structurally diverse and can be classified based on their structure. Table 9.1 lists the

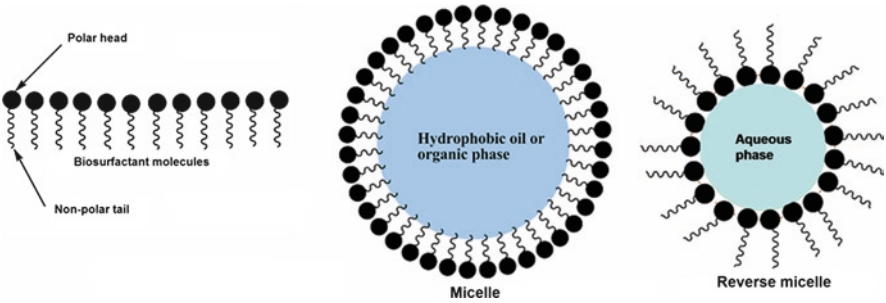

Fig. 9.1 General structure of a biosurfactant and its ability to form a micelle and reverse micelle

Table 9.1 Different types of biosurfactants and microbial source

Type of surfactant	Microorganism	References
Rhamnolipids	*Pseudomomas* sp., *P. aeruginosa*, *Serratia rubidea*	Mulligan (2005)
Sophorose lipids	*Candida apicola*, *C. bombicola*, *C. lipolytica*, *C. bogoriensis*, *Wickerhamiella domercqiae*, *C. Batistae*, *Pichia anomala*	Van Bogaert et al. (2007), Konishi et al. (2008), Thaniyavaran et al.(2008)
Trehalose lipids	*Arthrobacter paraffineus*, *Corynebacterium* spp., *Mycobacterium* spp., *Rhodococus erythropolis*, *Nocardia* sp.	Cooper et al. (1981b), Desai and Banat (1997), Mulligan (2005)
Cellobiose lipids	*Ustilago maydis*, *U. zeae*	Desai and Banat (1997)
Surfactin	*Bacillus subtilis*	Arima et al. (1968), Cooper et al. (1981a)
Mycolic acids	*Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Corynebacterium* species	Shimakata et al. (1984)
Corynomucolic acid	*Rhodococcus erythropolis*	Kretschmer et al. (1982)
Phospholipid	*Acinetobacter* sp., *Thiobacillus thiooxidans*, *Aspergillus* sp.	Beeba and Umbreit (1971), Kappeli and Finnerty (1979)
Viscosin	*P. fluorescens*	
Surfactin	*B. subtilis*	Arima et al. (1968)
Emulsan	*Acinetobacter calcoaceticus*	Rosenberg et al. (1979)
Liposan	*C. lipolytica*	Kappeli and Fiechter (1977),Cirigliano and Carman (1984)
Alasan	*Acinetobacter radioresistens*	Barkey et al. (1999)
Lipopolysaccharides	*Acinetobacter calcoaceticus (RAG1)*, *Pseudomonas* sp., *Candida lipolytica*	Mulligan (2005)
Lichenysin A, Lichenysin B	*B. licheniformis*	Mulligan (2005)

different types of biosurfactants along with their microbial sources. The most common classes of biosurfactants are discussed below.

Glycolipids. Glycolipids are the most common and most widely studied biosurfactants. They are nonionic and consist of a carbohydrate (mannose, rhamnose, etc.) head and a lipid tail. Rhamnolipids, sophorolipids, and trehalose lipids are best known examples of glycolipid-type biosurfactants (Desai and Banat 1997). Structurally, rhamnolipids are composed of one or two molecules of rhamnose sugar linked to one or two molecules of β-hydroxydecanoic acid. This group of biosurfactants is produced by a few *Pseudomonas* species and was first reported by Jarvis and Johnson (1949). Sophorolipids are mainly produced by a few yeasts of *Candida* species and consist of a dimeric glucose (also called sophorose) linked by a glycosidic bond through a hydroxyl group located at the penultimate position of an 18-carbon fatty acid (Van Bogaert et al. 2007; Daverey and Pakshirajan 2009). This type of biosurfactant occurs as a mixture of macrolactone and open-chain (free acid) forms and may be acetylated at the primary hydroxyl positions of the sophorose sugars. Gorin et al. (1961) were the first to report extracellular sophorolipids using the yeast *Torulopsis magnoliae* (later identified as *Candida apicola*). In comparison to other biosurfactants, e.g., rhamnolipids that are produced by pathogenic microorganisms, sophorolipids can be produced by non-pathogenic *Candida* species and in much larger quantities (Van Bogaert et al. 2007). In case of trehalose lipids, disaccharide trehalose sugar is linked to mycolic acid (a long chain α-branched-β-hydroxy fatty acid) and is mainly produced by *Mycobacterium*, *Nocardia*, *Corynebacterium*, *Rhodococcus*, and *Arthrobacter* species (Asselineau and Asselineau 1978; Kretschmer et al. 1982; Li et al. 1984; Cooper et al. 1981b; Desai and Banat 1997).

Lipopeptides and lipoproteins. Lipopeptides and lipoproteins produced by microorganisms are known for their antibiotic activity rather than their surfactant properties. Surfactin (a cyclic lipopeptide), produced by *Bacillus subtilis*, is the most thoroughly studied biosurfactant in this group (Cooper et al. 1981; Besson and Michel 1992) and was first reported by Arima et al. (1968).

Fatty acids, neutral lipids, and phospholipids. Several microorganisms produce fatty acids, phospholipids, or neutral lipids in large quantities when grown on *n*-alkanes and have been considered biosurfactants (Desai and Banat 1997). Fatty acids produced by microorganisms may be simple straight-chain fatty acids or may be complex in nature containing OH groups and alkyl branches (Rahman and Gakpe 2008). Mycolic acids are long-chain, β-hydroxy fatty acids substituted at the α-carbon atom with a moderately long aliphatic chain and are primarily produced by *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Corynebacterium* species (Shimakata et al. 1984). Corynomucolic acid is another example of a complex fatty acid biosurfactant produced by *Rhodococcus erythropolis* (Kretschmer et al. 1982). Triacylglycerols found in all eukaryotic cells (yeasts, molds, plants, and animals) and esters produced by *Acinetobatcer* sp. related bacteria such as *Moraxella* and some eukaryotic algae are examples of neutral lipids. *Acinetobacter* sp. strain HO1-N produces phosphatidylethanolamine, a phospholipid surfactant, when

grown on *n*-alkanes. *Aspergillus* sp. (Kappeli and Finnerty 1979) and *Thiobacillus thiooxidans* (Beeba and Umbreit 1971) have also been reported to produce phospholipids in large quantities.

9.2.2 Important Properties of Biosurfactants

Biosurfactants possess several properties similar to those of chemical surfactants. The most important properties are discussed below.

9.2.3 Surface and Interfacial Tension Reduction

Surfactants are evaluated based on properties such as surface or interfacial tension reduction and critical micelle concentration (CMC) (Parkinson 1985). Biosurfactant molecules tend to associate either with each other (micelle formation) or with surfaces between phases of different polarity (e.g., air/water, oil/water, or water/solid interfaces). Their effect on these interfaces is most readily seen as a reduction in surface tension. The surface tension at air/water and oil/water interfaces (often simply referred to as surface tension and interfacial tension, respectively) can be quantitatively determined by standard methods. As surfactant concentration is raised in an aqueous medium, the surface tension of the medium reduces, and eventually a point is reached at which surface tension of the medium takes up a minimal value and surfactant monomer begins to form micelle. The concentration of surfactant at which micelles begin to form is called CMC. Above the CMC, the surface tension remains constant, indicating that the interface is saturated and micelle formation has taken place in the bulk phase (Parkinson 1985). A lower CMC indicates that less surfactant is required to saturate the interface between either air–liquid or liquid–liquid and form micelles. Thus CMC is the most commonly used measure of surfactant efficiency. At concentrations higher than the CMC, it is hypothesized that additional surfactant molecules aggregate into micelles in the bulk phase and do not contribute to further significant changes on the interface. Values for surface and interfacial tension due to different biosurfactants reported in the literature are presented in Table 9.2. In general, biosurfactants are more effective and their CMC

Table 9.2 Properties of some important biosurfactants

Biosurfactant	Organism	Surface tension (mN/m)	CMC (mg/l)	Interfacial tension (mN/m)
Rhamnolipids	*P. aeruginosa*	29	0.1–10	0.25
Trehalolipids	*R. erythropolis*	32–36	4	14–17
Sophorolipids	*C. bombicola*	33	60	1.8
Surfactin	*B. subtilis*	27–32	23–160	1
Viscosin	*P. flurescens*	26.5	150	

Adopted from Desai and Banat (1997)

is about 10–40 times lower than that of chemical surfactants; i.e., compared with chemical surfactants, the quantity of biosurfactants required to reduce surface tension is substantially less (Desai and Banat 1997).

9.2.4 Emulsification and De-emulsification Activity

Stable emulsions with a lifespan of months to years can be produced using biosurfactants (Velikonja and Kosaric 1993). Also, biosurfactants can stabilize emulsions (emulsifiers) or destabilize them (de-emulsifiers). High molecular mass biosurfactants are, in general, better emulsifiers than low molecular mass biosurfactants (Muthusamy et al. 2008).

9.2.5 Biodegradability

One of the major advantages of biosurfactants over synthetic surfactants is their biodegradability; therefore, they are particularly suitable for bioremediation (Mohan et al. 2006). Zeng et al. (2007) studied the codegradation of synthetic surfactants (CTAB, Triton X-100, and SDS) and rhamnolipids with glucose by *Pseudomonas aeruginosa*, *B. subtilis* and compost microorganisms in liquid culture media. In addition to being recalcitrant to degradation by these microorganisms, CTAB inhibited organisms from utilizing the readily available carbon source (glucose) within the media. Although the nonionic surfactant Triton X-100 was found to be nontoxic to microorganisms and hence did not inhibit growth, it was recalcitrant to biodegradation. Anionic surfactant SDS also showed no toxicity toward microorganisms but could be codegraded as carbon source along with glucose. The biosurfactant rhamnolipid was a distinct type of surfactant that was nontoxic and well-degraded by *B. subtilis* and compost microorganisms; however, it could not be utilized by its producer organism, *P. aeruginosa*. Biodegradability of sophorolipids produced by *Candida bombicola* was studied by different authors and is reported to be a readily biodegradable biosurfactant (Hirata et al. 2009; Lo and Ju 2009).

9.2.6 Low Toxicity

Microbial surfactants are generally considered as less toxic or nontoxic products and, therefore, may be better suited for pharmaceutical, cosmetic, and food applications when compared with chemical surfactants (Flasz et al. 1998; Muthusamy et al. 2008). Poremba et al. (1991) found that a synthetic anionic surfactant (Corexit) displayed an LC_{50} against *Photobacterium phosphoreum* ten times lower than rhamnolipids, hence demonstrating the higher toxicity of the chemical-derived surfactant.

Similarly, sophorolipids displayed low cytotoxicity toward human fibroblasts, human keratinocytes cell-line HPK II, and normal human epidermal keratinocytes (Krivobok et al. 1994; Otto et al. 1999; Hirata et al. 2009).

9.3 Remediation of Contaminated Soil and Water Using Different Physical, Chemical, and Biological Techniques

Soil and water contaminated with hazardous chemicals is a major public health and environmental concern worldwide. In general, hazardous chemicals are generated as by-products of industries such as petrochemicals, pulp and paper, chemical manufacturing, mining, and others.

Several physical, chemical, and biological remediation techniques have been successfully applied by researchers, but more often a single remediation strategy may not provide sufficient removal of contaminants from soil or water; therefore, combinations of two or more remediation techniques are often used for achieving optimal results. Short descriptions of these techniques are provided below.

9.3.1 Physical Techniques

The objective of physical remediation techniques is to separate the contaminants from soil or water. Contaminants thus removed are generally subjected to ex situ treatment. Excavation and soil vapor extraction are the two most widely used physical techniques. Air sparging and soil washing using surfactant or cosolvent flushing are some of the other physical techniques used for treating contaminated soil and water. Generally, these techniques are costly and require further treatment (Wirthensohn et al. 2009).

9.3.2 Chemical Techniques

Oxidizing agents such as H_2O_2, Fenton's reagent, permanganate, ozone, and sodium persulfate are widely used for remediation of contaminated soil and water. Selection of oxidant depends on the nature and type of contaminant, level of remediation required, viability of oxidant delivery, soil conditions, and hydrogeology of the site. The greatest advantages of chemical techniques are the relatively rapid treatment time and the ability to treat contaminants present at high concentrations. Nonselectivity and capital cost are among the major disadvantages of these methods. In addition, many oxidizing agents are highly reactive, which lead to high reaction temperatures with the risk of explosion. Field scientists must be adequately trained in the use of these chemicals.

9.3.3 Biological Techniques or Bioremediation

Bioremediation is an emerging and promising technology for the treatment of contaminated soil and water. The primary requirements for bioremediation are that the microorganisms used should be capable of surviving in the contaminated environment and also efficient in degrading the contaminant. Therefore, bioremediation can be effective only where environmental conditions permit microbial growth and activity. Sometimes environmental parameters must be manipulated to allow microbial growth and degradation of contaminants. Depending on the organism and the contaminant(s) of concern, bioremediation can be either aerobic or anaerobic. *Pseudomonas*, *Alcaligenes*, *Sphingomonas*, *Rhodococcus*, and *Mycobacterium* are a few of the microbial genera, which are reported to degrade contaminants such as pesticides and hydrocarbons under aerobic conditions. Many of these bacteria use the contaminant as the sole source of carbon and energy. The major advantage of this technique is its cost-effectiveness and permanent removal of pollutant by complete mineralization of the contaminant. Furthermore, the technique can be designed to be noninvasive and leave the ecosystem intact. Bioremediation can address low concentrations of contaminants where cleanup by physical or chemical methods would not be feasible. When compared with conventional methods, the major drawbacks of this technique are longer process times and low predictability of the system. Selected bioremediation methods are described below.

In situ bioremediation. This method is generally the most desirable option due to low cost and less disturbance, since treatment occurs in-place, thus avoiding excavation and transport of contaminants. In situ treatment is limited by depth of affected soil that can be treated. In many soils, effective oxygen diffusion for desirable rates of bioremediation extends to a range of only a few centimeters to about 30 cm, although depths of 60 cm and greater have been effectively treated in some cases (Vidali 2001). To address this issue, oxygen can be pumped to lower depths via dissolution of air, peroxide, ozone, etc. into influent water. The most important in situ land treatments are briefly described further.

Bioventing is the most common in situ treatment (Lee and Swindoll 1993; Vidali 2001) and involves supplying air and nutrients through wells to contaminated soil to stimulate the proliferation of indigenous bacteria. These organisms will work to decompose the contaminants, while negative pressure removes waste vapors. Bioventing employs low air flow rates and provides only the amount of oxygen necessary for biodegradation while minimizing volatilization and release of contaminants to the atmosphere (Leeson and Hinchee 1997; Vidali 2001). It works for simple hydrocarbons and can be used where the contamination is deep below the surface.

Biosparging. In this in situ technique, air (or oxygen) is injected below the water table to increase groundwater oxygen concentration and enhance the rate of biological degradation of contaminants by bacteria. This system increases mixing in

the saturated zone, thereby facilitating contact between oxygen and soil and groundwater. The ease and low cost of installing small-diameter air injection points allows considerable flexibility in the design and construction of the system.

Bioaugmentation. This in situ technique frequently involves the addition of microorganisms that are indigenous or exogenous to the contaminated sites. Two factors limit the use of added microbial cultures in a land treatment unit (1) nonindigenous cultures rarely compete well enough with an indigenous population to develop and sustain useful population levels and (2) most soils with long-term exposure to biodegradable waste have indigenous microorganisms that are effective degraders, if the land treatment unit is well managed.

9.3.3.1 Ex Situ Bioremediation

The ex situ method involves excavation or removal of contaminated soil. The soil is subsequently treated in surface units.

Landfarming is a simple technique in which contaminated soil is excavated and spread over a prepared bed and periodically tilled until pollutants are degraded. The goal is to stimulate indigenous microorganisms and facilitate aerobic degradation of contaminants. In general, the practice is limited to the treatment of surficial soil layers (e.g., 10–35 cm). Since landfarming has the potential to reduce monitoring and maintenance costs as well as clean-up liabilities, this technique has received much attention as a remediation alternative.

Composting involves combining contaminated soil with nonhazardous organic amendments such as manure or agricultural wastes. The presence of these organic materials supports the development of a rich microbial consortium and elevated temperatures. By this technique, hydrocarbon contaminants are degraded both biologically and chemically.

Biopiles Fahnestock et al. (1998) are a hybrid of landfarming and composting. Engineered cells are constructed as aerated composted piles and used for the treatment of surface contamination with petroleum hydrocarbons. This is a refined version of landfarming that tends to control losses of the contaminants by leaching and volatilization. Biopiles provide a favorable environment for the action of indigenous aerobic and anaerobic microorganisms.

Bioreactors. Slurry reactors or aqueous reactors are used for ex situ treatment of contaminated soil and water. A slurry bioreactor may be defined as a vessel used to create a three-phase (solid, liquid, and gas) mixing condition to increase bioremediation rate of soil-bound and water-soluble pollutants. Owing to the creation of optimized environmental conditions and high mass transfer rates in bioreactors, the degradation rate of contaminant(s) is substantial. However, the system requires physical excavation of soil before applying the bioreactor system. In addition, reactor conditions (pH, N levels, gas release, etc.) must be monitored closely.

9.4 Bioremediation of Contaminated Soil and Water Using Biosurfactants

Owing to their ability to reduce surface/interfacial tension between two phases and form micelles, biosurfactants play an important role in the bioremediation of soil and water contaminated with hydrophobic pollutants and also in the removal of heavy metals from contaminated soil. The role and mechanism of biosurfactants in bioremediation of sites contaminated with various pollutants are discussed below.

9.4.1 Hydrocarbons

Hydrocarbons are organic compounds, which consist solely of hydrogen and carbon. They can be classified as saturated hydrocarbons, unsaturated hydrocarbons, cycloalkanes, and aromatic hydrocarbons. They are used primarily as energy sources; however, if present in soil or water, they are toxic to the environment and human health due to their mutagenicity, carcinogenicity, and tendency to bioaccumulate in the food chain.

9.4.2 Polycyclic Aromatic Hydrocarbons

PAHs are a class of organic compounds containing two or more fused aromatic rings. Naphthalene, phenanthrene, anthracene, and pyrene are examples of PAHs. Some PAHs have been shown to possess carcinogenic characteristics. Their release and subsequent accumulation in terrestrial environments is cause for concern (Cerniglia 1992). Therefore, soils containing these substances require remediation (Shuttleworth and Cerniglia 1995; Kanaly and Harayama 2000).

The low water solubility of PAHs increases their sorption to surfaces and limits their bioavailability to microorganisms, which is a barrier to effective bioremediation of sites contaminated with PAHs. Biosurfactants can enhance microbial growth on bound substrates by desorbing them from surfaces or by increasing their apparent water solubility (Marcoux 2000). Surfactants that dramatically lower interfacial tension are particularly effective in mobilizing bound hydrophobic molecules and making them available for biodegradation. Low-molecular-weight biosurfactants that have low CMCs increase the apparent solubility of hydrocarbons by incorporating them into the hydrophobic cavities of micelles (Miller and Zhang 1997; Ron and Rosenberg 2002). Several authors have studied the effect of different biosurfactants to increase biodegradation of PAHs (Schippers et al. 2000; Arun et al. 2008; Gottfried et al. 2010; Sponza and Gok 2010). Figure 9.2 shows the mechanism of PAH removal by biosurfactants. The initial step is micelle formation, where the hydrophobic portion of the biosurfactant attaches to the PAH through hydrophobic interactions and forms micelles containing the PAH. The micelle contacts the cell membrane which is highly hydrophilic and increases membrane porosity for the

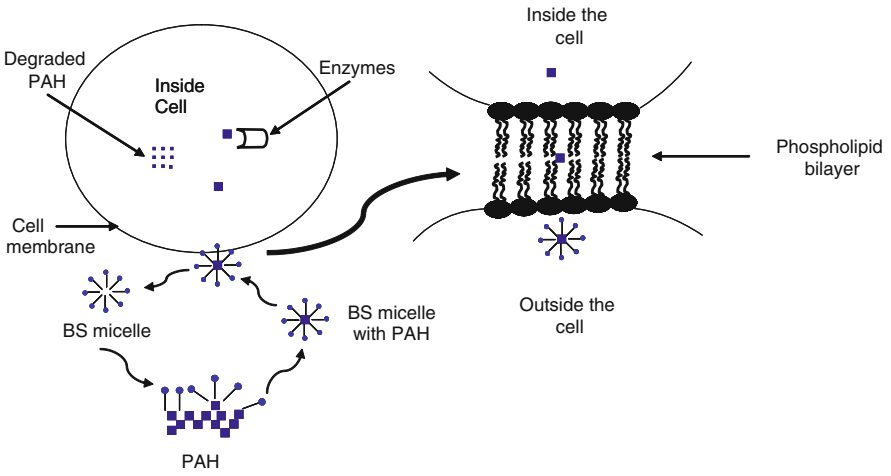

Fig. 9.2 Mechanism of PAH degradation by microorganisms in the presence of a biosurfactant

PAH to enter the cell. Once inside the cell, the PAH is attacked by the necessary enzymes and are degraded (Schippers et al. 2000). Another possible mechanism of biosurfactant action involves modification in cell hydrophobicity. If a biosurfactant attaches to the cell membrane and changes cell hydrophobicity, it will facilitate direct contact between cells and the PAH, which leads to PAH uptake by the cell and its biodegradation. Alasan (a high-molecular-weight bioemulsifier complex of an anionic polysaccharide and proteins) produced by *Acinetobacter radioresistens* KA53 increases the apparent aqueous solubilities of phenanthrene, fluoranthene, and pyrene up to 6.6-, 25.7-, and 19.8-fold, respectively (Barkey et al. 1999). Addition of rhamnolipid biosurfactant increases the apparent aqueous solubility of phenanthrene and overall degradation by at least 20% when combined with salicylate or glucose in liquid solution when compared with solutions that contained salicylate or glucose with no biosurfactant (Gottfried et al 2010).

Recently, Sponza and Gok (2010) studied the degradation of PAHs in a petrochemical wastewater in a continuous stirred tank reactor and reported that the addition of rhamnolipid increased the removal efficiencies of PAHs from 72 to 80%. Rhamnolipid treatment significantly increased the degradation of five- and six-ring PAHs. Arun et al. (2008) isolated a biosurfactant-producing *Pseudomonas* strain from oil-contaminated soil and found that it degraded 90% of pyrene. Table 9.3 summarizes the literature on biosurfactant-aided degradation of different hydrocarbons by microorganisms.

9.4.3 Petroleum Hydrocarbons

Petroleum hydrocarbons include alkanes, cycloalkanes, aromatics, PAHs, asphaltenes, and resins. Alkanes are represented by the formula C_2H_{2n+2} (where n

Table 9.3 Hydrocarbons degraded by different microorganisms using biosurfactants

Hydrocarbon	Microorganism	Biosurfactant	% Biodegradation	References
Naphthalene	*Pseudomonas* sp.	Rhamnolipid	100	Vipulanandan and Ren (2000)
Phenanthrene	*P. putida*	Sophorolipid	99.4	Schippers et al. (2000)
Pyrene	*Pseudomonas* sp.	Multicomponent surfactant consisting of protein and polysaccharides	92.3	Arun et al. (2008)
Acenaphthene			28	
Fluorene			24.4	
Phenanthrene	*P. putida*	Rhamnolipid (0.25 g/l)	86	Gottfried et al. (2010)
		Rhamnolipid (1 g/l)	91	
		Rhamnolipid (5 g/l)	92	
Mixture of PAH containing petrochemical industrial wastewater	Activated sludge	Rhamnolipid	80	Sponza and Gok (2010)
Phenanthrene	*Pseudomonas aeruginosa* coculture with *P.* strain R	Rhamnolipid	50	Dean et al. (2001)
Mixture of hydrocarbons	Soil population	Sophorolipid	95	Oberbremer et al. (1990)
Gasoline	Soil sample	Rhamnolipid (0.1%)	67	Rahman et al. (2003)
		Rhamnolipid (1%)	78	

is the number of carbons and $2n+2$ is the number of hydrogens) and are sometimes referred to as aliphatic compounds. Low-molecular-weight alkanes are the petroleum hydrocarbons most readily degraded by microorganisms. As chain length and the amount of branching increases, resistance of the compound to microbial attack increases (Mulligan 2005).

Biosurfactants are reported to increase the biodegradation of petroleum hydrocarbons by mechanisms similar to that of PAHs (Zhang and Miller 1992; Shreve et al. 1995). Zhang and Miller (1992) demonstrated that rhamnolipids at a concentration of 300 mg/l increased mineralization of octadecane from 5 to 20% compared with that of controls. Rahman et al. (2003) examined the bioremediation of *n*-alkanes in a petroleum sludge, which contained an oil and grease content of 87.4%. The following degradation efficiencies in 10% sludge were reported after 56 days with the addition of a bacterial consortium plus nutrients and rhamnolipids: C8–C11 alkanes: 100%; C12–C21: 83–98%; C22–C31: 80–85%, and C32–C40: 57–73%. Although biodegradation rate decreased with increase in chain length, rates were significantly high even for C32–C40 compounds, thus indicating the benefit of rhamnolipid addition in enhancing biodegradation of these highly hydrophobic compounds. Whang et al. (2008) investigated the potential application of two biosurfactants, surfactin and rhamnolipid, for enhanced biodegradation of diesel-contaminated soil and water in a series of bench-scale experiments. In batch experiments with diesel/water, addition of 40 mg/l surfactin significantly enhanced diesel biodegradation up to 94%, compared with 40% with no surfactin addition. Similarly, the addition of rhamnolipid to diesel/water systems in the range of 0–80 mg/l substantially increased the biodegradation of diesel from 40 to 100%. These results confirmed the utility of the biosurfactant in enhancing both efficiency and rate of diesel biodegradation in diesel/water and diesel/soil systems. Recently, Lai et al. (2009) studied microbial ability to degrade total petroleum hydrocarbons (TPHs) from soil by two biosurfactants (surfactin and rhamnolipid) compared with that of synthetic surfactants (Tween 80 and Triton X-100). The authors reported that biosurfactants exhibited much higher TPH removal efficiency than did the synthetic surfactants. By using 0.2% each of rhamnolipids, surfactin, Tween 80, and Triton X-100, TPH removal for soil contaminated with ca. 3,000 mg TPH/kg dry soil was 23, 14, 6, and 4%, respectively. For soil contaminated with ca. 9,000 mg TPH/kg soil, the removal efficiency increased to 63, 62, 40, and 35%, respectively, due to the surfactants. TPH removal efficiency also increased with increase in biosurfactant concentration (from 0 to 0.2%); however, with increase in contact time from 1 to 7 days, efficiency did not vary significantly.

9.4.4 Pesticides and Herbicides

The pesticides dichlorodiphenyltrichloroethane (DDT), 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), plasticizers, pentachlorophenol, and polychlorinated biphenyls, among others are examples of

halogenated aromatic compounds. Their stability and toxicity are cause for concern for the environment and public health. The halogenated aliphatic compound, position, and number of halogens are important in determining both rate and mechanism of biodegradation (Mulligan 2005). Pesticides and herbicides have poor aqueous solubility and biosurfactants are suggested to increase their aqueous solubility, thereby increasing degree of biodegradation.

Rhamnolipids at 4 g/l have shown the capability to increase the mineralization of 4,4′ chlorobiphenyl by an acclimated culture of *Alcaligenes eutrophus* up to 213 times above that of the control (Robinson et al. 1996). Both rhamnolipids and Triton X-100 increased biodegradation of trifluralin and coumaphos in liquid cultures and soil slurries by microbial consortia isolated from contaminated cattle dip (Mata-Sandoval et al. 2001). A study conducted by Wattanaphon et al. (2009) demonstrated that biosurfactants were more active in solubilizing pesticides compared with synthetic surfactants. The authors examined the ability of a glucolipid-type of biosurfactant from an isolated strain *Burkholderia cenocepacia* BSP3 to enhance pesticide solubility in comparison with those of synthetic surfactants (nonionic surfactant Tween 80 and anionic surfactant SDS). Solubilization efficiency of three pesticides with distinct water solubility, viz. methyl parathion, ethyl parathion, and trifluralin, was shown to be dependent on surfactant and its concentration. The biosurfactant from *B. cenocepacia* BSP3 substantially enhanced the apparent solubility of the three pesticides when the concentration was increased to 2 CMC; however, an increase in the biosurfactant concentration within the range of 2–10 CMC did not significantly enhance pesticide solubility. Under the conditions tested, biosurfactant from *B. cenocepacia* BSP3 was more efficient than Tween 80 and SDS in enhancing pesticide solubilization. Singh et al. (2009) isolated a *Pseudomonas* sp. (ChlD) from agricultural soil by enrichment culture technique in the presence of chlorpyrifos, capable of producing biosurfactant (rhamnolipids) and degrading chlorpyrifos. They evaluated the ability of different concentrations of rhamnolipids ranging from 0.02 to 0.04 g/l in M-9 medium to improve partitioning of chlorpyrifos to the aqueous phase. Chlorpyrifos solubility in the aqueous phase increased from 2.5% in controls to >87% in medium supplemented with 0.04 g/l of a biosurfactant preparation. The quantity of chlorpyrifos decreased concomitantly with an increase in biosurfactant concentration from 0.02 to 0.04 g/l, indicating significant increase in aqueous phase partitioning of chlorpyrifos. In the absence of rhamnolipids, 91.7% of chlorpyrifos was degraded, whereas in the presence of the biosurfactant, over 98% of chlorpyrifos degradation was achieved.

The isomers of hexachlorocyclohexane (HCH) are a class of pesticides, which are reported to be highly toxic and carcinogenic and to be endocrine disrupters. The spatial arrangement of chlorine atoms in different HCH isomers and low aqueous phase solubility contribute to their persistence in the environment. Rhamnolipids produced from an isolated strain of *P. aeruginosa* WH-2 improved aqueous solubility of HCH isomers, suggesting the potential role of biosurfactants in bioremediation of pesticides and herbicides (Sharma et al. 2009).

9.4.5 Heavy Metals

Soils contaminated with metals are the result of direct contact with industrial discharge, improper disposal of wastes, spills and failure of land disposal facilities. Metals such as lead (Pb), chromium (Cr), cadmium (Cd), mercury (Hg), arsenic (As), copper (Cu), nickel (Ni), and zinc (Zn) have been detected in many sites (Forstner 1995; Kim and Vipulanandan 2006). Metals, unlike many hazardous organic constituents, cannot be degraded or readily detoxified. The existence and fate of the metals in soil is a matter of concern not only because of their potential impact on microbial communities but also because of the potential for groundwater contamination and hence toxicological impacts on human health (Kim and Vipulanandan 2006). Metal contaminants in the environment are usually tightly bound to colloidal particles and organic matter. This represents a major constraint on their removal using currently available in situ remediation technologies (Juwarker et al. 2008).

Biosurfactants can improve metal removal; however, the mechanism differs from solubilization, mobilization, sorption and emulsion formation, etc., which have been postulated for hydrocarbon removal. Mulligan et al. (1999b) studied metal removal with surfactin and described possible mechanisms by which biosurfactants remove metals from soil. They suggested that the biosurfactant must be forming complexes through direct contact with the sorbed metal contaminants before detachment from the soil. The biosurfactant reduces the interfacial tension between the soil colloid and metal, which enables the removal of the metal from the soil surface. The biosurfactant micelle makes a strong complex with metal, which prevents readsorption of the metal to the soil.

Due to the anionic nature and complexation ability of rhamnolipids, they aid in the removal of metals, including ions of Cd, Cu, lanthanum (La), Pb, and Zn from soil (Tan et al. 1994; Herman et al. 1995; Ochoa-Loza 1998; Mulligan 2005).

Biosurfactants could be added to soil during ex situ soil washing processes. Due to the foaming property of biosurfactants, metal–biosurfactant complexes can be removed by the addition of air to cause foaming and then the biosurfactant can be recycled via precipitation by reducing solution pH to 2. Neilson et al. (2003) studied Pb removal by rhamnolipids. A 10 mM solution of rhamnolipid removed about 15% of soil Pb after ten washes. High levels of Zn and Cu did not impact lead removal.

Rhamnolipids have been added to mining ores to enhance metal extraction (Dahrazma and Mulligan 2004). Batch tests were performed at room temperature, and using a 2% rhamnolipid concentration, 28% (2.6 mg) of the Cu was extracted. Addition of 1% NaOH enhanced removal up to 42% (3.8 mg) for a rhamnolipid concentration of 2%, but removal decreased at higher surfactant concentrations. This study confirmed that pH plays an important role in removal of metals by biosurfactants. Sequential extraction studies were performed to characterize the mining ore and to determine the types of metals being extracted by the biosurfactants. Approximately 70% of the Cu was associated with the oxide fraction, 10% with the

carbonate, 5% with the organic matter, and 10% with the residual fraction. After washing with 2% biosurfactant at pH 6 for 6 days, 50% of the carbonate fraction and 40% of the oxide fraction were removed.

The effect of pH on metal removal by rhamnolipids was studied by Asci et al. (2007). At low pH (4.3–5.8), the rhamnosyl moiety of the rhamnolipids is at least 50% unchanged and rhamnolipids form liposome-like vesicles. Between pH 6.0 and 6.6, rhamnolipids form either lamella-like structures (6.0–6.5) or lipid aggregates (6.2–6.6). When the rhamnosyl moiety is negatively charged above pH 6.8, micelles form. This suggests that, for better performance of rhamnolipids, the pH should be above 6.8. The optimal Cd(II) recovery efficiency (70.6–70.2% from kaolinite) was achieved using rhamnolipids by adjusting initial pH to 6.8–7.0 (Asci et al. 2007). Similarly, rhamnolipids removed a maximum 98.8% of Zn from Na-feldspar at pH 6.8 (Asci et al. 2008). On the other hand, low pH is more suitable for the sophorolipids type of biosurfactant, because above pH 7.0 the surfactant solution becomes a milky white solution with precipitate. After a single washing of soil with solutions containing sophorolipids (4% alone, 4% plus 0.7% HCl, and 4% plus 1% NaOH), Zn removal efficiency was 4, 16 and 7%, respectively (Mulligan et al. 1999a).

Kim and Vipulanandan (2006) evaluated the removal of Pb from water and contaminated soil (kaolinite) using a biosurfactant produced from vegetable oil. Over 75% of the Pb was removed from 100 mg/l contaminated water at ten times the CMC, and the biosurfactant:Pb ratio for optimal removal in the system was found to be 100:1. FTIR spectroscopy indicated that the carboxyl group of the biosurfactant was involved in the removal. Dahrazma and Mulligan (2007) evaluated the performance of rhamnolipids in a continuous flow configuration (CFC) for removal of Cu, Zn, and Ni from sediments collected from Lachine Canal, Canada, to simulate a flow-through remediation technique. In this configuration, a rhamnolipid solution was passed through the sediment sample within a column at a constant rate. The removal from sediments was 37% of Cu, 13% of Zn, and 27% of Ni when rhamnolipid without additives was applied. Adding 1% NaOH to 0.5% rhamnolipid improved Cu removal by up to four times compared with 0.5% rhamnolipid alone.

Juwaker et al. (2008) isolated the di-rhamnolipid biosurfactant by *P. aeruginosa* strain BS2 and further assessed the potential of isolated biosurfactants as a washing agent for metal removal from multimetal contaminated soil (Cr, 940 mg/kg; Pb, 900 mg/kg; Cd, 430 mg/kg; Ni, 880 mg/kg; Cu, 480 mg/kg). Between tap water and rhamnolipids, rhamnolipids proved very efficient in the mobilization of metal from contaminated soil; within 36 h of the leaching study, di-rhamnolipid when compared with tap water facilitated a 13-fold higher removal of Cr from the soil, whereas removal of Pb and Cu were 9–10 and 14-fold higher than with tap water only. On the other hand, leaching of Cd and Ni from the spiked soil was 25-fold higher using rhamnolipids compared with the use of tap water. These results showed that leaching behavior of the biosurfactant was different for different metals. Table 9.4 summarizes the studies that have successfully demonstrated the removal of metals from contaminated systems using biosurfactants.

Table 9.4 Removal of different metals by biosurfactants

Metal	Biosurfactant	% Removal	References
Cd	Rhamnolipid (0.5%) foam	73.2	Mulligan and Wang (2006)
	Rhamnolipid (0.5%) liquid solution	61.7	Mulligan and Wang (2006)
	Rhamnolipid	71.9	Asci et al. (2007)
	Rhamnolipid (0.1%) pH 8	92	Juwarker et al. (2008)
	Biosurfactant isolated from marine bacterium	97.66	Das et al. (2009)
Ni	Rhamnolipid (0.5%) foam	68.1	Mulligan and Wang (2006)
	Rhamnolipid (0.5%) liquid solution	51	Mulligan and Wang (2006)
Pb	Rhamnolipid (10 mM solution)	15	Neilson et al. (2003)
	Rhamnolipid (0.1%) pH 8	88	Juwarker et al. (2008)
	Biosurfactant isolated from marine bacterium	100	Das et al. (2009)
Zn	Rhamnolipid (12%)	19.5	Mulligan et al. (1999a)
	Sophorolipid (4%)	15.8	Mulligan et al. (1999a)
	Sophorolipid (4%) with HCl (0.7%)	100	Mulligan et al. (1999a)
	Surfactin (0.25%) with NaOH (1%)	22	Mulligan et al. (1999b)
Zn from Na-feldspar	Rhamnolipids (25 mM), pH 6.8	98.83	Asci et al. (2008)
Cu	Rhamnolipid (12%)	25	Mulligan et al. (1999a)
	Surfactin (1%) with NaOH (1%)	70	
	Rhamnolipid (2%)	28	Dahrazma and Mulligan (2007)

9.5 Recent Advances in Bioremediation Processes Using Biosurfactants and Future Prospects

9.5.1 Use of Immobilized Microorganisms and Contaminants

In situ biosurfactant production and bioremediation of contaminated sites using immobilized microorganisms are viewed to be more economical and more advantageous than many conventional chemical and physical methods. For instance, the use of immobilized cells can overcome adverse environmental conditions that threaten microbial survival and can also prevent direct contact of introduced microorganisms with the autochthonous microbial community (Barreto et al. 2010). In addition, immobilization facilitates the monitoring of microbial metabolism and offers the possibility of repeated use of the cells (Cassidy et al. 1996).

Recently, Barreto et al. (2010) entrapped spores of *B. subtilis* LAMI008 (biosurfactant-producing bacteria) in chitosan beads and these beads were cross-linked with glutaraldehyde (to improve the stability of the chitosan beads) for *n*-hexadecane biodegradation and eventual biosurfactant recovery. The entrapped cells degraded almost 100% of *n*-hexadecane (1%) in a medium supplemented with 1% glucose within 48 h. The number of viable cells inside the beads was maintained throughout the experiment, and the released biosurfactant was not depleted as carbon source. Entrapment of the bacterial spores in chitosan beads was reported to overcome problems with stability, storage, and long-term cell viability encountered with vegetative cells. This approach can potentially be utilized for biodegradation of complex compounds by entrapping spores of different species of biosurfactant-producing bacteria. Mahanty et al. (2009) reported a novel approach for biodegradation of PAHs by encapsulating pyrene into alginate beads for controlled release of pyrene and its subsequent biodegradation by *Mycobacterium frederiksbergense*. Although this strain is not a biosurfactant producer, biodegradation of immobilized contaminants using biosurfactant-producing microorganisms could also be tested.

9.5.2 Novel Strains for Producing Biosurfactants

The primary requirement for microorganisms in bioremediation is the ability to grow and/or survive in the contaminated environment and efficiently degrade the contaminant. Although several microorganisms have been reported for their effectiveness in bioremediation, there is still a need for effective biosurfactant-producing microorganisms, particularly for developing in situ bioremediation processes.

9.6 Applications of Biosurfactants in Agriculture

In agriculture, microbial surfactants are used for hydrophilization of heavy soils to obtain good wettability and to achieve even distribution of fertilizers. They also prevent the caking of certain fertilizers during storage and promote wetting, spreading, and penetration of pesticides (Kosaric et al. 1987). There are very few reports available on the use of biosurfactants in agriculture, and therefore, more detailed study is required to evaluate their potential.

9.7 Conclusion

Biosurfactants have shown potential for remediation of contaminated soil and water. Although the published literature suggests that biosurfactants are easily biodegradable and are less toxic compared with synthetic surfactants, most studies are

limited only to ex situ treatment using rhamnolipids. There is, therefore, a need to explore the potential of other biosurfactants such as sophorolipids and novel biosurfactants that may possess superior properties than those of rhamnolipids, as well as to successfully develop suitable biosurfactant-based in situ treatment processes.

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Chapter 10
Bioaugmentation-Assisted Phytoextraction Applied to Metal-Contaminated Soils: State of the Art and Future Prospects

Thierry Lebeau, Karine Jézéquel, and Armelle Braud

Abstract Bioaugmentation-assisted phytoextraction is a promising method for accelerating the cleanup rate of soils contaminated by metals. On average, bioaugmentation increases metal accumulated by plant shoots by factors of about two (metal concentration) and five, as a result of higher bioaccessibility of metals in soils, with few obvious differences between effects by bacteria or fungi (e.g., plant growth-promoting rhizobacteria and arbuscular mycorrhizal fungi). Metal bioaccessibility is always controlled by microbial siderophores as well as organic acids and surfactants. In cases of excess concentrations, fungi immobilize metals, in contrast to bacteria. Unfortunately, the typically low inoculant survival rate may impair bioaugmentation efficiency. In this chapter, microbial inoculant formulations and management are addressed, as well as strategies for selecting the most relevant plant–microorganism couples for optimum phytoextraction of soil metals. In environments subject to variable conditions, ecological engineering approaches may help in attaining maximal efficiency. Experiments at field-scale are reported, and environmental effects of the technique are discussed. Finally, future prospects are addressed with the main question being how maximal concentrations and amounts of metals in plants can be attained.

10.1 Introduction

Unlike organic pollutants, whose degradation can be undertaken in the very soil matrix to be cleaned up, toxic metal remediation implies removal (e.g., by solubilization or complexation). Nonpoint source contamination involving moderate concentrations of metals along extensive surfaces is less studied than is point source contamination.

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_10,

However, when analyzing the situation in Europe and in the USA, approximately 100,000 ha of land is contaminated by heavy metals (Lewandowski et al. 2006) as a result of both repeated applications of fertilizers and pesticides containing trace metals, along with atmospheric deposits. Although metal concentrations encountered are lower than those recorded in industrial sites, they are sufficiently high to generate a risk for the environment and humans through food chain contamination.

In situ soil phytoextraction is a "soft technology" allowing for sustaining crop production following treatment. Phytoextraction could even generate an economic benefit for farmers (Lewandowski et al. 2006) when several options for management of contaminated agricultural soils are compared. Among the limitations of this technique (e.g., shallow soils are reached by roots and poor metal translocation from roots to shoots), its slow rate resulting from low bioaccessibility of metals is often considered to be the main limiting factor (Khan et al. 2000).

To increase metal availability to plants, various synthetic compounds have been tested (see review of Evangelou et al. 2007), but several limitations were observed: low biodegradability; toxicity to plants, microorganisms, and nematodes; lixiviation risk and high cost. A promising alternative consists in optimizing the synergistic effect of plants and microorganisms (Glick 2003) by coupling soil bioaugmentation (Lebeau 2010) with phytoextraction, a process termed rhizoremediation (Kuiper et al. 2004). Phytoextraction can be improved by increasing plant biomass as a result of plant growth-promoting rhizobacteria (PGPR) effect or by facilitating metal uptake with enzymes, siderophores, organic acids, or biosurfactants synthesized by the microbial inoculant (Jing et al. 2007). Plants also play a major role as nutrient suppliers for maintaining microbial growth and activity. Data published until 2008 were extensively reviewed by Lebeau et al. (2008) where metal bioaugmentation-assisted phytoextraction was described in a technological viewpoint, i.e., efficiency and control of the metal phytoextraction in environments subject to variable conditions. Since then, new results have confirmed previously published data, i.e., there is an average increase in metal concentration and total amount extracted by plants by factors of two and five, respectively.

After reviewing the basic mechanisms governing plant–microorganism relationships, this chapter addresses some practical advice on implementing on-site metal phytoextraction-assisted bioaugmentation from solid matrices such as soil and sediment. Ecological engineering could be a relevant approach for in situ metal phytoextraction-assisted bioaugmentation to attain maximal efficiency and control in environments subject to variable conditions. Methods are also reviewed to allow for a thorough understanding of mechanisms involved in this technique. Finally, future prospects are suggested.

10.2 Mechanisms Driving Metal Extraction in Plant–Microorganism Systems

The low quantities of metals extracted from soil by plants as well as the slow rate of extraction are primarily a consequence of low metal accessibility. Indeed, plant-available metals often account for less than one percent of the total metal content

of a soil (Whiting et al. 2001; Braud et al. 2006b). This brings us to the issue of defining bioavailability vs. bioaccessibility. Definitions were provided by Semple et al. (2004): "a bioavailable compound [was defined] as that which is freely available to cross an organism's cellular membrane from the medium the organism inhabits at a given time," whereas a bioaccessible compound corresponds to "what is available to cross an organism's cellular membrane from the environment, if the organism has access to the chemical."

10.2.1 Metal Bioaccessibility as a Result of Microbial Mechanisms

In general, microbial processes solubilizing metals increase their bioaccessibility, whereas those immobilizing them reduce bioaccessibility (Gadd 2001). The balance between metal mobilization and immobilization represents the amount of metals available for uptake by the plant. This quantity varies depending on the metal, microorganism, environment, and physicochemical conditions such as pH, CEC, and organic matter content (Kayser et al. 2001).

In a bioaugmented process coupled with phytoextraction, solubilization provides a route for metal removal from soils manifesting itself in different forms (Gadd 2004):

Heterotrophic leaching. Chemoorganotrophic microorganisms acidify the local environment by efflux of H+ pumps due to the action of H+-ATPases, or by accumulation of CO_2 due to respiration. Leaching can also be due to the ability of microorganisms to synthesize metabolites (siderophores, biosurfactants, and organic acids) (Braud et al. 2006b; Di Simine et al. 1998; Wasay et al. 1998; Mulligan et al. 1999b). The most efficient acids include citric acid, which is known to solubilize Ni and Zn (Castro et al. 2000; Valix et al. 2001), and oxalic acid for Pb contained in pyromorphite (Sayer et al. 1999). Biosurfactants can also play a role in metal mobilization; examples include surfactin produced by *Bacillus subtilis* and rhamnolipids by *Pseudomonas aeruginosa*. Biosurfactants complex the free metallic form, which increases metal desorption from the solid phase and also mobilizes metal sorbed to the solid phase by the formation of micelles (Miller 1995). Surfactin and rhamnolipids have been reported to mobilize Cu from the organic fraction of soil (Mulligan et al. 1999a). Siderophores are low-molecular-weight molecules produced in soil at a few µmol/L of soil solution (Bossier et al. 1988). Having a strong affinity for Fe (about 10^{30} for the affinity constant), siderophores can, nonetheless, complex other metals in solution such as Cu, Ga, Mn, Ni, and Zn, albeit with lower affinity (Höfte et al. 1993; Braud et al. 2009a). Braud et al. (2006a) demonstrated that siderophore-producing bacteria inoculated in soil enhanced lead and chromium mobility. Pots bioaugmented with *Aspergillus niger* and *P. aeruginosa* contained higher concentrations of Cr (0.08 and 0.25 mg/kg dw soil) and Pb (0.25 and 0.3 mg/kg dw soil) in the exchangeable fraction (i.e., extraction with $MgCl_2$) in comparison with nonbioaugmented soil where neither Cr nor Pb was detected. Diels et al. (1999) have shown that siderophore produced by

Alcaligenes eutrophus CH34 markedly increases the availability of Cd, Pb, and Zn by complexation (Gadd and White 1993).

Autotrophic leaching. Acidophilic, chemolithotrophic bacteria, which fix carbon dioxide, obtain energy from the oxidation of Fe(II) to Fe(III), and sulfur to H_2SO_4.

Biomethylation. This reaction occurs for Ag, Hg, Pb, Se, Sn, and Te. The methylated metals are biologically more mobile than their corresponding elemental forms (Gadd 1993).

Redox reactions. The reduction of Fe and Mn oxides by microbes results in strong metal absorption and increases the mobility of metals in the soil.

10.2.2 Mechanisms Controlling Metal Uptake by Plants

Depending on metal bioaccessibility, metallic form, and plant status, metals are absorbed by roots as soluble forms or complexed to organic matter. Metal mobilization within the rhizosphere occurs in exchange for proton extrusion, or secretion of phytosiderophores or organic acids. Nonetheless, no correlation has been found between hyperaccumulation and rhizospheric soil pH (McGrath et al. 1997) or with hyperproduction of root exudates (Zhao et al. 2001). Rhizospheric microorganisms have been shown, in certain situations, to stimulate root exudation (Groleau-Renaud et al. 2000). Despite the strong influence of root exudates on metal mobility (Mench et al. 1987), no study has shown the indirect impact of soil microorganisms on phytoextraction rate through stimulation of root exudation.

Once absorbed by the root, metals in ionic form can be immobilized by complexation with carbonates, sulfates, and phosphates contained within plant cells (Raskin et al. 1994). Yet, complexation with some organic compounds such as histidine, nicotianamine, and citrate tends to facilitate metal transport in xylem, while metallothionein and phytochelatin complexation tends to immobilize metals in root vacuoles (Clemens et al. 2002). Indeed, several phytoextraction studies have shown that citric acid and oxalic acid amendment increased metal accumulation as well as translocation factor in the plant (James and Bartlett 1984; Srivastava et al. 1998).

Translocation rate varies as a function of metallic species; some are easily translocated toward leaves (Cd, Co, Mn, Ni, and Zn), while others accumulate in the roots (Al, Cr, Cu, Fe, and Pb) (Baker et al. 1994; Pulford and Watson 2003). Hyperaccumulator plants, where the translocation factor is greater than 1, tend to sequester less quantity of metals in their vacuoles in comparison with nonhyperaccumulators (Singh et al. 2003). Although it cannot be applied to all Ni hyperaccumulator species, metal exposition of Ni hyperaccumulator *Alyssum* sp., *Streptanthus polygaloides*, and *Berkheya coddii* increased histidine concentration in xylem, which increased metal transport and translocation (Kramer et al. 1996; Smith et al. 1999; Salt and Kramer 2000).

Plant physiological status and stress response also play a role in phytoextraction rate. The plant's strategy for managing metal toxicity involves synthesis of metal chelators such as phytochelatins or metallothioneins to chelate and detoxify the

metal absorbed (Zenk 1996). The expression of glutamylcysteine synthetase, a precursor of phytochelatins, was shown to enhance Cd accumulation and tolerance in *Brassica juncea* (Zhu et al. 1999). The synthesis, by bacteria, of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which degrades ACC, an ethylene precursor that can block root elongation if the plant is stressed, is a means of reducing stress to plants (Glick 2003).

10.3 Practical Issues and Recommendations with Phytoextraction-Assisted Bioaugmentation

The following two definitions of bioaugmentation should be kept in mind in the current discussion: (1) the definition given by El Fantroussi and Agathos (2005), i.e., "the technique for improvement of the capacity of a contaminated matrix (soil or other biotope) to remove pollution by the introduction of specific competent strains or consortia of microorganisms" and (2) a broader one suggested by Dejonghe et al. (2001), i.e., "this approach corresponds to increasing the metabolic capabilities of the microbiota present in the soil. In that respect, bioaugmentation corresponds to an increase in the gene pool and, thus, the genetic diversity of that site." Soil bioaugmentation can be applied to plant nutrition in the following aspects: increase in plant growth, control of phytopathogens (biocontrol), improvement of soil structure, mineralization of organic pollutants, and bioaccumulation or biolixiviation of inorganic pollutants (van Veen et al. 1997). Bioaugmentation techniques have been widely used with the genus *Rhizobium* and *Frankia* and other nonsymbiotic microorganisms to increase plant growth, including *Azospirillum*, *Azotobacter*, *Burkholderia*, *Gluconacetobacter*, *Herbaspirillum*, and *Klebsiella* (Kennedy et al. 2004), and *Bacillus*, *Pseudomonas*, *Aspergillus*, and *Penicillium* (Khan and Khan 2002). New bioaugmentation processes have been developed to increase metal transfer to plants, using arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) (Glick 1995). This new technique, termed rhizoremediation (Kuiper et al. 2004), is mainly used to enhance biodegradation of organic pollutants by providing a host for degrading bacteria. The effects of nonsymbiotic and symbiotic bacteria on phytoextraction efficiency have been extensively reviewed by Lebeau et al. (2008).

10.3.1 Mutualistic and Symbiotic Relationships with Plants

Microorganisms used in augmented phytoextraction can have a mutualistic or symbiotic relationship with the host plant. Symbiotic microorganisms can be exosymbionts, i.e., residing outside the roots, mesosymbionts, or endosymbionts such as AMF, which belong mainly to the Glomales order and can colonize 97% of plant species (Abbott and Robson 1991). Depending on the plant–AMF association and soil conditions, AMF have a protective role toward metal toxicity and tend to

increase plant tolerance but decrease metal accumulation in shoots (Leyval et al. 2002). Indeed, Hovsepyan and Greipsson (2004) suggest the use of a fungicide during phytoextraction of Pb by maize to avoid metal immobilization by AMF. Due to their high surface of sorption, AMF can chelate metals in the cells due to the production of glomalin (González-Chávez et al. 2004). Nonetheless, several studies have shown that AMF bioaugmentation increased Cd accumulation in bean and maize (Guo et al. 1996), alfalfa (El-Kherbawy et al. 1989), clover (Joner and Leyval 1997), and soybean (Heggo et al. 1990). Results differ as a function of plant species; for example, *Glomus intraradices* inoculation increased Pb concentration in *Agrostis capillaris* roots, but not in *Zea mays* (Malcova et al. 2003). The success of phytoextraction assisted with AMF is variable also as a function of plant–microbial association, physicochemical properties of the soil, metal availability, inoculation conditions, and root density (Leyval et al. 1997). Furthermore, the use of AMF in phytoextraction can be criticized, since most hyperaccumulator plants belong to the Brassicaceae family and are typically nonmycorrhizal and are, therefore, not associated with AMF.

Bioaugmentation with nonsymbiotic microorganisms could ensure a higher success for rhizoremediation, since bacterial colonization would be less dependent on the plant–microbe association. The best known microbial group, i.e., PGPR, can influence metal extraction either indirectly by increasing plant growth rate due to (1) P and K solubilization, (2) production of indole acetic acid (IAA), a phytohormone that increases plant growth, (3) production of ACC deaminase, which degrades ACC, an ethylene precursor that can block root elongation if the plant is stressed, and (4) production of siderophores, or directly by increasing metal mobility with microbial metabolites (Glick 2003).

In most contaminated soils, P and K are poorly available nutrients. The inoculation of K- and P-solubilizing bacteria can increase both plant growth due to nutrient release and extraction of metals associated with P or K (Halstead et al. 1969). Some bacteria such as *B. subtilis* SJ-101, which produces IAA, increased *B. juncea* metal tolerance and improved Ni shoot extraction by 1.5-fold (Zaidi et al. 2006). Experiments with bacteria that naturally synthesize ACC deaminase such as *Kluyvera ascorbata* SUD165 did not show any improvement in metal extraction (Burd et al. 1998; Belimov et al. 2001), while plants engineered with bacterial ACC deaminase genes experienced increased metal extraction (Cd, Co, Cu, Mg, Ni, Pb, and Zn) in comparison with nonmodified plants (Grichko et al. 2000).

Metal contamination is often linked with Fe deficiency and plant stress, which implies development of chlorotic symptoms in plants growing on contaminated sites (Imsande 1998). Bacterial siderophores can act as an Fe supplier for the plant, increasing its growth and metal resistance, and increasing metal mobility in the soil (Bar-Ness et al. 1992). Siderophores are known as powerful Fe chelatants and can also complex other divalent and trivalent ions (Visca et al. 1992; Duffy and Defago 1999), thus increasing metal mobility in the soil. For example, desferrioxamine B, a siderophore produced by *Streptomyces*, is able to mobilize Pb sorbed on goethite (Dubbin and Ander 2003), and siderophores produced by *Burkholderia cepacia* were able to desorb Cd from goethite (Mirabello 2006). Several studies have

reported that bioaugmentation with siderophore-producing microorganisms increases plant growth and metal phytoextraction (Höflich and Metz 1997; Whiting et al. 2001; Braud et al. 2009b).

According to Rajkumar et al. (2009), endophytic bacteria could be more suitable than nonendophytic PGPR for assisted phytoextraction because of their mutualistic relationship with the plant, ensuring better root colonization than would PGPR. However, the mechanisms for metal mobilization and/or plant growth promotion are the same as for PGPR. Endophytic bacteria colonizing roots of *Brassica napus* and producing IAA, siderophores, and ACC deaminase have been reported to facilitate Pb uptake and plant growth (Sheng et al. 2008).

10.3.2 Microbial Consortia

Some studies have used microbial consortia to enhance metal phytoextraction rate. A bacterial consortium adapted to selenium increased the bioconcentration factor in *B. juncea* by three, in comparison with the control, but decreased phytoextraction efficiency due to reduction of biomass production (Lampis et al. 2009). Another study showed that coinoculation of AMF and *B. cereus* increased Cd, Cr, Mn, and Ni phytoextraction by *Trifolium repens* (Azcón et al. 2009). Usually consortia are more efficient than pure cultures, as they do not lose their remediation ability and they allow the use of noncultivable microorganisms (Gentry et al. 2004).

10.3.3 Factors Impairing Bioaugmentation Success

Limiting factors for the success of bioaugmentation include the decrease of bacterial survival after inoculation due to abiotic and biotic stress factors, and restricted mobility due to microbial size and attachment (Gentry et al. 2004; Lebeau 2010). The latter factor encourages the use of bacteria instead of fungi due to their smaller size and greater mobility. In order to increase bioaugmentation efficiency, several techniques have been developed: encapsulation of cells within a matrix, use of genetically engineered microorganisms to transfer genes of interest in indigenous microorganisms, rhizosphere bioaugmentation as an ecological niche for inoculated microorganisms, use of ultramicrobacteria and adhesive-deficient bacteria, and use of surfactants to enhance mobility.

The main factors ensuring success of soil colonization is the efficiency of the inoculation method and the selection of bacteria based on their rhizocompetence. The rhizocompetence of a bacteria can be expressed as its ability to (1) mobilize iron, (2) reduce nitrate and carry out denitrification, and (3) use specific carbon sources and nutrients present in root exudates (Curl and Truelove 1986; Latour et al. 1996). Bacterial growth strategy is also an important factor for soil colonization; fast-growing bacteria (i.e., r strategists) are mostly found in young and immature

roots, while slow-growing bacteria (K strategists) grow mostly on mature roots (De Leij et al. 1995; Nacamulli et al. 1997). Phenotypical variations of microorganisms can also favor root colonization due to their adaptability as a result of root maturation, as shown for root colonization of *Arabidopsis thaliana* by *Pseudomonas brassicacearum* (Achouak et al. 2004). Siderophore production and Fe mobilization provide a competitive advantage for soil colonization by bacteria toward antagonists or predators. Statistical analyses have shown that the most competitive rhizospheric populations belong to the same siderotype (Latour et al. 2003).

10.3.4 Genetically Engineered Microorganisms

Most engineered microorganisms applied in phytoremediation have been used to increase metal resistance or adsorption and/or accumulation, but not specifically to enhance metal mobility. Most research has been focused on engineered microorganisms to increase microorganism resistance to metals, to accumulate As, and to volatilize Hg and Se (Valls and Lorenzo 2002). A strain of *B. cepacia* holding the Ni resistance system genes of *Ralstonia metallidurans* increased root accumulation of Ni in *Lupinus luteus*, but not shoot Ni accumulation (Lodewyckx et al. 2001).

Increasing microbial metabolite production by genetic modification can enhance phytoextraction rates. A siderophore-overproducing mutant of *Enterobacter*,NBRI K28 SD1, has been reported to increase Zn extraction by *B. juncea* shoots (Kumar et al. 2008).

10.4 Plants

Most plants used in phytoextraction should resist metals, should possess a high growth rate and a developed root system, and must be able to efficiently translocate metals from root to shoot. (McGrath et al. 2002). Several approaches have been developed to enhance phytoextraction efficiency, including use of hyperaccumulators, use of high-biomass plants assisted by chemical or biological techniques, and use of fast-growing trees and genetically engineered plants.

10.4.1 Hyperaccumulators vs. High-Biomass Species

The major limiting factor in phytoextraction is remediation time, which can reach several decades. A reasonable and economically viable length, i.e., lower than 5 years (Khan et al. 2000), implies the selection of species adapted to their environment and effective on lightly contaminated sites.

The choice of hyperaccumulator species vs. high-biomass species depends on soil parameters, metal speciation, and climatic conditions. Depending on the plant

and metallic species, predictive models have shown that the hyperaccumulator *Thlaspi caerulescens* is the most efficient plant for Zn phytoextraction, while the nonhyperaccumulator *Nicotiana tabacum* is the most effective for Cd remediation (Liang et al. 2009). Most hyperaccumulator species are low-biomass plants with a low growth rate, which can be a disadvantage for remediation of a large area as compared with using high-biomass species (Baker et al. 2000; Reeves and Baker 2000). For example, *Z. mays* cultivated on the same area as *T. caerulescens* can extract three times more Cd, Cu, and Zn than the latter species because of higher biomass production (Keller and Hammer 2005). Another study compared the efficiency of different plant species on soil contaminated with 2,500 mg/kg Pb and showed that *Z. mays* could extract twice as much Pb than the best *B. juncea* cultivar and almost three times more than *Thlaspi rotundifolium* (Huang and Cunningham 1996). Therefore, numerous high-biomass plants have been used in phytoextraction processes, including *Avena sativa*, *B. juncea* and *B. napus*, *Helianthus annuus*, *Hordeum vulgare*, *Pisum sativum*, and *Z. mays*.

Fertilization can increase the yield of hyperaccumulator species and increase their potential for phytoextraction. With light NPK fertilization *B. coddii* biomass increased twofold to reach 22 tons/ha/year, and Ni concentration in shoots increased with higher rates of N amendments (Brooks and Robinson 1998). Fertilizer amendments must be adjusted for each plant species, since excessive N fertilization can reduce Cd and Zn extraction by *T. caerulescens* and excessive P fertilization can even reduce its biomass (Sirguey et al. 2006). Furthermore, the use of P fertilizer can decrease metal availability and precipitate Pb in soil as pyromorphite (Scheckel et al. 2005).

10.4.2 Mobilization of Metals by Plants: The Role of Siderophores and Phytosiderophores

Plants are able to mobilize or immobilize metals by secreting root exudates, protons, phytosiderophores (PS), or organic acids (Kinnersley 1993; Wenzel et al. 2003). PS are synthesized by strategy II plants, mostly graminaceous, under mugenic or avenic acid forms or nicotianamines in the case of Fe or Zn deficiency (Gries et al. 1995). Strategy I plants use proton exudation, Fe(III) reductases, and Fe(II) transporters for Fe uptake. Yet, PS are also able to complex other metals such as Cd, Ni, and Zn (Awad and Romheld 2000; Shenker et al. 2001). Plant exudates also contain low-molecular-weight organic acids (citric, malic, oxalic), which can mobilize Al, Ca, and Fe and increase their phytoextraction rate either by formation of metallic complexes or by reducing rhizosphere pH (Haynes 1990; Hinsinger et al. 2003).

A strong relationship exists between Fe status of the plant and siderophore production in the rhizosphere (Yang and Crowley 2000). Several plants such as *Lycopersicon esculentum* (Duss et al. 1986), *Dianthus caryophyllus*, and *H. vulgare* (Duijff et al. 1991), *Vigna radiata* and *Z. mays* (Sharma and Johri 2003a, b),

Arachis hypogaea (Jurkevitch et al. 1988), and *A. thaliana* (Vansuyt et al. 2007) are able to use fluorescent *Pseudomonas* siderophores as an Fe supplier. It has been reported that bacterial siderophore ferrioxamine B (FOB) can be preferentially used by plants in comparison with Fe–EDTA or Fe–PS and is rapidly used in the Fe-deficient zones of *Cucumis sativus* (Wang et al. 1993). On the other hand, mugenic acid is more efficient than FOB for Fe uptake and translocation by *Z. mays* (Crowley et al. 1992), and PS are also more efficient than rhizoferrin, a fungal siderophore, for Fe uptake in wheat and barley (Shenker et al. 2001). These studies show that strategy I plants like *C. sativus* accumulate mostly siderophore–Fe complexes, while strategy II plants such as *Z. mays* are able to produce PS and more readily absorb PS–Fe complexes.

Few studies have shown the role of siderophore-producing microorganisms in enhanced phytoextraction. *Pseudomonas* and *Enterobacter* increased Zn extraction twofold by *T. caerulescens* (Whiting et al. 2001). Maize phytoextraction of Pb and Cr increased by 5.4 and 3.8, respectively, by siderophore-producer *P. aeruginosa*, and Cr shoot extraction was enhanced by 5.2 after *R. metallidurans* bioaugmentation (Braud et al. 2009b). Another study reported that hydroxamate-type siderophores secreted by *Streptomyces tendae* increased Cd and Fe uptake by 60% in *H. annuus* shoots (Dimkpa et al. 2009).

10.4.3 Plant Development

Except iron status, plant development stage is an important factor for determining the length of the remediation process and to reduce the treatment cost of biomass after phytoextraction. Indeed, the concentration factor from soil to shoot for Cd extraction by *Solanum nigrum* was lower at mature stage than at flowering stage (Wei et al. 2006). Another study reported that As concentration decreased in *Pteris vittata* fronds after 2 months growth, while the concentration in roots increased. It was concluded that young plants, with higher metabolic activities, were more suitable for phytoextraction (Gonzaga et al. 2007).

Fast-growing trees are considered good candidates for phytoextraction due to their deep root systems, adaptability to different substrates, and high biomass production (Pulford and Watson 2003). Their rate of transpiration being high, the transfer of metal from root to shoot is facilitated in trees; for example, *Salix* can transpire 19,000 L of water in one summer (Hinchman et al. 1998). An economic study has shown that *Salix* can be adapted for the remediation of agricultural soil contaminated by Cd (Lewandowski et al. 2006).

10.4.4 Genetically Engineered Plants

Most engineered plants used in phytoextraction have been employed for phytovolatilization of mercury and selenium. Several strategies have been used for genetic improvement of the plant, such as alteration of uptake system specificity and

increasing chelatant production (Krämer and Chardonnens 2001). Understanding of the *P. vittata* As hyperaccumulation mechanism has led to the production of a modified *Arabidopsis* plant able to extract threefold more As than the nonmodified plant (Dhankher et al. 2002). Increasing production of chelant precursors glutathione and PC synthase increased metal tolerance and accumulation in the plant (Bennett et al. 2003; Gisbert et al. 2003; Martínez et al. 2006). Furthermore, transfer of animal metallothionein genes to *A. thaliana*, *Nicotiana* sp., and *Brassica* sp. increased plant tolerance to Cd but did not increase Cd accumulation (Karenlampi et al. 2000), while introduction of metallothionein from *P. sativum* into *A. thaliana* increased Cu uptake by roots (Evans et al. 1992). Another strategy increased transpiration rate by suppressing a transpiration inhibitor such as abscisic acid; a *B. juncea* mutant with this characteristic extracted twofold more Pb than did the wild plant (Gleba et al. 1999).

10.5 Practical Recommendations for Selection of Plant–Microorganism Couples and Implementation of the Bioaugmentation-Phytoextraction Technique

10.5.1 Strategy for Choosing the Most Relevant Plant–Microorganism Couples

In order to attain the greatest degree of metal extraction by plants, the optimal plant–microbial inoculant combination must be selected. The microbial inoculant should encompass the following properties: (1) sufficient metal mobilization from soil or sediment, (2) adequate survival rate, (3) plant protection against metal toxicity, and (4) plant growth stimulation. Regarding choice of plant, the main criteria are (1) biomass per soil surface area, (2) metal translocation and accumulation, (3) root colonization depth, (4) number of metals extracted by a single plant species, and (5) amount and composition of rhizodeposits available to support microbial growth. The complex interrelationships between these two partners complicate the selection scheme. Selected aspects of phytoextraction-assisted bioaugmentation are summarized in Table 10.1. Some are detailed below.

The major problem with symbiotic microorganisms lies in their inability to modify the associations and that some plants are not involved in symbiosis, such as hyperaccumulators belonging to the Brassicaceae family (e.g., *B. juncea* and *T. caerulescens*), which are typically nonmycorrhizal (Brown et al. 1994; Kumar et al. 1995). Conversely, regarding the host plant playing the dominant role in the symbiotic relationship, much more attention should be paid to host plants than to AMF (Chen et al. 2003) which are nonhost-specific symbionts. Among nonsymbiotic microorganisms, endophytic bacteria have been shown to tolerate higher concentrations of Ni than do rhizospheric bacteria (Idris et al. 2004). Unfortunately, most endophytic bacteria are cultivation-independent. Yet, only biostimulation can be suggested, not bioaugmentation (Zhuang et al. 2007). Facultative endophytes such

Table 10.1 Characteristics of microorganism–plant interactions (from the reviews of Lebeau et al. 2008; Badri et al. 2009; Glick 2010; Lebeau 2010)

		Positive aspects	Negative aspects
Microorganisms Type of association with the plant	Symbiotic microorganisms		Inability to modify the associations except AMF, which are nonhost-specific symbiontsMicroorganisms
	Endophytic bacteria	They have been shown to tolerate higher concentrations of metal than rhizospheric bacteria	Most are cultivation-independent (biostimulation can be used, not bioaugmentation)
	Nonsymbiotic microorganisms	Some have been shown to be nonplant-specific and can thus be associated with numerous plants	Some only colonize certain plants
Beneficial microbial activity to plant growth	AMF	Solubilize phosphorusPhenolic defense system with the formation of thiol compounds. Such phytochelatins can detoxify metals	
	PGPR (phytohormones especially auxins such as IAA, cytokinins, and gibberellins)	Enhance various stages of plant growth: IAA, one auxin phytohormone, enhances lateral and adventitious rooting, root exudation and increases tolerance to salt	
	ACC deaminase (cleaves the plant ethylene precursor ACC)	Lowers plant ethylene levels in a stressed plant. Avoids inhibition of root elongation, nodulation, and auxin transport. Delays speed aging, senescence, and abscissionPositive effect shown when flooding, presence of variety of different metals, phytopathogens, and drought	Ethylene shows a positive effect during the first stage of plant growth

	Atmospheric N_2 fixing		Minor component of the benefit that the bacterium provides to plants
	Siderophores	Can sequester iron from the soil and provide it to plants cells which can take up the bacterial siderophore–iron complex	
Beneficial microbial activity to metal accumulation by plant	Siderophores	Help plants acquire iron in the presence of overwhelming amounts of other metals targeted for phytoextraction purposes	
	Biosurfactants		
	Organic acids		
Plants			
Plant type	Hyperaccumulators belonging to the Brassicaceae family		Typically nonmycorrhized plants
	Symbiotic plants		The host plant playing the dominant role in symbiosis, much more attention should be paid to host plants than to AMF
Beneficial plant activity to microorganisms	Rhizodeposits (amino acids, organic acids, proteins, sugar, etc.)	Organic acids (more than sugars and amino acids) could be used as substrates to evaluate the ability of inocula to colonize the plant roots	Full composition of most plant exudates is unknown, preventing selection of relevant microorganisms with purified exudates prior to definitively selecting the microorganism–plant associations
	Signal molecules		Specific plant signal could be received by each rhizobacterium making the microorganism–plant selection more complex

as *Pseudomonas fluorescens* G10 and *Microbacterium* sp. G16 can, however, be used (Sheng et al. 2008), as they could colonize rhizosphere soil and plant tissue. Some microorganisms have been shown to be nonplant-specific (de Souza et al. 1999) and can thus be associated with numerous plants. Nonetheless, some PGPRs only colonize certain plants (Zhuang et al. 2007). For example, *Pseudomonas* extracted from the rhizosphere of maize and inoculated in the same rhizosphere showed better colonization compared to *Pseudomonas* originating from the rhizosphere of another plant (Benizri et al. 1997). Facing these constraints, Kuiper et al. (2001, 2004) were the first to suggest selecting strains indigenous to a host plant's rhizosphere, calling this selection procedure "rhizo-directed strain selection."

Regarding plants, cocultivation of two or more species is suggested for two key reasons: (1) some plants, particularly those belonging to the *Brassicaceae* family, cannot be associated directly with AMF. However, hyperaccumulators *T. caerulescens* and *Sedum alfredii* exhibit higher Zn uptake in the presence of mycorrhized maize (Wu et al. 2006b). These systems, associating nonmycorrhized hyperaccumulators with mycorrhized plants, offer prospects for optimizing phytoextraction yields (Khan 2005); and (2) most soils are contaminated with several metals – 70% of contaminated sites in the USA are affected by at least two metals (Forstner 1995), while most plant species only accumulate one or two metal species. Associations of multiple plant species, with the aim of collectively accumulating the metal mixture, should be undertaken and optimized. Unfortunately, most studies of multicontaminated soils restrict themselves to analyzing the botanical composition of the site without testing the relevancy of different plant associations in extracting several metals (Diez Lazaro et al. 2006; Regvar et al. 2006; Li et al. 2007). The cropping of relevant plant associations should be experimented with, where each plant is associated with the most appropriate microorganism(s).

Soil metal concentration and fertility are additional important parameters to be considered regarding the plant–microorganism selection, since bioaugmentation may decrease metal uptake by plants above certain concentrations. AMF–plant symbiosis can be used primarily for less-contaminated soils (Audet and Charest 2007a). For example, maize, which has established symbiosis with the AMF *Acaulospora mellea,* accumulates more Cu in shoots and roots compared to the nonmycorrhized plant for Cu concentrations less than 400 mg/kg of soil (Wang et al. 2007). A similar Cu concentration threshold was observed by Andrade et al. (2010) for a Jack bean–*Glomus etunicatum* association. No shift from positive to negative effect of bioaugmentation on metal extraction was shown in the range of metal concentrations tested. For example, the effects of 800 mg Pb/kg were compared to 400 mg/kg with isolates of *P. fluorescens* G10 and *Microbacterium* sp. G16 (Sheng et al. 2008); concentrations ranged from 0 up to 580 mg/kg for Ni and 0 up to 9,780 mg/kg for Ni with soil inoculated with *Bradyrhizobium* sp. (*Vigna*) (Wani et al. 2007).

PGPR effects on plant growth can become impaired when plants are cultivated under optimal and stress-free conditions (Glick 2010).

The time and space to grow plants are also limiting factors when plant–microorganism selection is undertaken. Although microorganisms must ultimately be tested with plants, a preselection could consist in cultivating microorganisms with artificial root exudates, as they play a key role in the establishment and

growth of microorganisms in the rhizosphere. Their compositions would mimic different plant species and physiological states. The composition of root exudates originated from some plants are already well known (Chaudhry et al. 2005), as are the variability of their composition under the influence of various parameters such as photoperiod cycle (Melnitchouck et al. 2005) and cultivars (Kush and Dadarwal 1981; Krotzky et al. 1988). Thus, new microbial culture media whose composition is similar to root exudates are suggested. This preselection would greatly reduce the time required for final selection, and therefore, more combinations could be tested. With this selection scheme strategy, metal phytoavailability in soil could be determined by single-extraction procedures with chemical extractants.

10.5.2 Preculture Conditions of Microbial Inoculants

The ability of microorganisms to proliferate in soil after bioaugmentation has not been sufficiently taken into account, often impairing microbial survival (Lebeau 2010). In addition to nutrient availability in soil, microbial persistence and activity are related to the phenotypical characteristics of the microorganisms and inoculation procedures (Thompson et al. 2005) before soil bioaugmentation, encompassing both inoculant preculture and packaging. A "priming effect" was first announced by Bingemann et al. (1953). Basically, priming is predisposing an isolate or population of microorganisms to future conditions in which they are designed to perform a function (Singer et al. 2005). The difference between "priming" and "activated soil" is as follows: soil priming aims at directly selecting microorganisms in the soil to be cleaned up. Activated soil first relies on the "priming" of a fraction of soil by the addition of the pollutant(s) with the aim of selecting relevant microorganisms and secondly at bioaugmenting the soil to be cleaned up. Activated soil serves at the same time of the inoculant, carrier, and source of nutrients without extracting the degraders from the soil (Gentry et al. 2004). The main advantages of these techniques are as follows: (1) a pool of useful complementary microorganisms is available; (2) a pool of cultivable and noncultivable microorganisms is available; (3) no steps of extraction and culture of microorganisms are necessary; and (4) better microbial survival, since soil serves as a carrier for microorganisms. Preculture media such as soil extracts whose compositions are closer than that of artificial culture media could also help at increasing microbial survival after soil bioaugmentation (Lebeau et al. 2002).

10.5.3 Selection and Bioaugmentation with Consortia: More Efficient than Pure Culture?

In theory, the use of microbial consortia instead of monoculture is more relevant as consortia can perform more complicated tasks, acting synergistically with others

and thus enduring more changeable and harsh conditions in polycontaminated environments (Verstraete et al. 2007; Brenner et al. 2008). "Natural" and "artificial" microbial consortia can be used in bioaugmentation. In the former case, the global performances may be optimal, but assemblages can maintain homeostasis, since members generally do not outcompete one another and do not exhaust the resources in their environments, and they can generally sustain harsh conditions as the result of selection over a long period (Brenner et al. 2008). Although microbial communities comprising consortia are constantly changing, the subtle equilibrium between the different populations making up the consortium is generally not broken, resulting in a high resiliency of these systems. Only the proportions of the populations making up the consortia may change. For example, a minority population can become the most active population during nutrient limitation (LaPara et al. 2002). Hence, microbial communities are most probably the basis of microbial resource management in the domain of environmental biotechnology. Opposite to natural consortia, artificial consortia could be attractive by creating assemblages by gathering previously selected microorganisms such as superbugs (Singer et al. 2005). Nonetheless, the long-term behavior and destiny of such a consortium is, however, unpredictable.

Some microbial consortia, e.g., *Glomus mosseae* and *Brevibacillus* (Vivas et al. 2003, 2006), *G. intraradices* and *Glomus spurcum* (Toler et al. 2005), *G. mosseae* and *A. niger* (Medina et al. 2006), *Microbacterium saperdae*, *Pseudomonas monteilii* and *Enterobacter cancerogenes* (Whiting et al. 2001), a mixture of six rhizobacteria (de Souza et al. 1999), and *Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus mucilaginosus* (Wu et al. 2006b), were tested with the aim of enhancing metal phytoextraction. No outstanding differences in quantity of metals accumulated by plants were demonstrated between pure cultures and microbial consortia (Lebeau et al. 2008).

10.5.4 Microbial Inoculant Formulations and Management

Numerous studies use bacteria introduced in liquid culture stage (free cells), which does not guarantee proper distribution of bacteria in the soil profile, shelf life, or activity (Mrozik and Piotrowska-Seget 2010). Inoculant formulations have been tested to enhance inoculum survival. Artificial immobilization is a relevant means to stabilize inocula (i.e., growth and activity) (Labana et al. 2005; Plangklang and Reungsang 2009; Siripattanakul et al. 2009) as biofilms do for natural consortia. Seeds of *Alyssum murale* were inoculated by soaking with bacterial suspensions added to methylcellulose (Abou-Shanab et al. 2006). Willow cuttings from annual shoots were inoculated with *Paxillus involutus* in a peat–vermiculite substrate (Baum et al. 2006). Other formulations with peat have been experimented (Belimov et al. 2004; Wu et al. 2006a). Soil bioaugmentation with immobilized *P. aeruginosa* cells supplied with skim milk increased Cr and Pb uptake by maize shoots by a factor of 5.4 and 3.8, respectively (Braud et al. 2009b). A commercial inoculum composed of

rhizobacterial strains (*Azospirillum lipoferum*, *Arthrobacter mysorens*, *Agrobacterium radiobacter*, and *Flavobacterium* sp.) was tested (Belimov et al. 2004).

In many cases, it can be assumed that the main limiting factor impairing cell survival and growth of inoculants is the amount of soil substrates available for microorganisms, rather than the microbial ability to survive, as shown by Braud et al. (2009b) who compared skim milk supply with cell immobilization in Ca-alginate matrix. Yet, cell immobilization was able to protect inoculants from grazing, interspecific competition (Willaert and Baron 1996; Lebeau 2010), and environmental stresses such as toxicity of metals (Braud et al. 2007). Addition of substrates to soil that are specifically metabolized by the targeted microorganisms may contribute to their survival (Duquenne et al. 1999; Kuiper et al. 2001; Braud et al. 2006a, 2007).

Various inoculum sizes were tested (see review of Lebeau et al. 2008 for details) but do not seem to be correlated with rates of metal extracted by plants (Rai et al. 2004; Braud et al. 2006b). As discussed above, one can assess that soil oligotrophy may impair establishment of additions of biomass. Reinoculation aims at disturbing the balance of the ecosystem to the benefit of the inoculum, with varied success.

In the case of bioaugmentation via seed soaking, the microbial inoculant must be densely associated with seeds. Alternatively, soil may be bioaugmented at the time of planting or before, in particular, with AMF (Lebeau et al. 2008).

10.5.5 Culture Duration and Planting Density

Regarding culture duration, experiments can be divided into short term (typically 1 week up to 2 months), performed mainly under laboratory and greenhouse conditions, and long term (approximately 4–6 months) in field conditions. The literature rarely mentions whether or not time of harvesting corresponds to maximal accumulation of the target metal in plants. Furthermore, kinetic studies are very scarce, whereas the need of nutrients useful to plants varies greatly over the growing period. In a field experiment with the fern *Pityrogramma calomelanos* associated with rhizobacteria or rhizofungi, Jankong et al. (2007) showed that the amount of As in fronds was slightly higher at 12 weeks than at 6 weeks, while the amount in roots was lower (decrease by a factor of up to four). In a nonbioaugmented control, higher As accumulation in roots and fronds was recorded in 6 weeks. A similar finding by Chen et al. (2006) occurred for As and ^{238}U accumulation by another fern (*Pteris vittata* L.) mycorrhized with different *Glomus* species and harvested after 8 and 12 weeks.

Rhizospheric biomass is also strongly altered according to plant growth stage. *B. juncea* populations decrease as follows: seedling stage > flowering stage > tillering stage when associated with *B. juncea* (Wu et al. 2006a). Once inoculant is supplied to the soil, the additional amount of metal available for plants is reached within a few days. Peak values of water-soluble Pb and Cd in soil solution were reached 48 and 72 h, respectively, after soil inoculation with *Burkholderia* sp.

J62 (Jiang et al. 2008). The values were associated with a 2 pH unit decrease. Similar observations were made by Braud et al. (2006b) for crop soils contaminated by Cr and Pb and bioaugmented by *P. fluorescens*, *P. aeruginosa*, or *A. niger*.

Planting density also influences the acquisition of elements by plants from the environment. The "target-neighbour" cocropping approach is useful to determine the effect of planting density on metal uptake (Shann 1995). This method is based on the ecological concept that states that as density increases, competition for essential resources intensifies.

10.5.6 Experiments on Field Scale

Only limited experiments have been implemented in field conditions (Belimov et al. 2004; Citterio et al. 2005; Zaidi et al. 2006; Farwell et al. 2007; Jankong et al. 2007). Belimov et al. (2004) conducted experiments on both laboratory and field scales. The positive rhizobacterial effect on Cd and Pb uptake by barley was shown in both pot and field experiments. Similar results were found by Jankong et al. (2007) with the fern *Pityrogramma calomelanos* bioaugmented with rhizobacteria in greenhouse and field experiments for As phytoextraction. Validation on field scale of the results obtained in the laboratory should be intensified in the next few years.

10.5.7 Economic Aspects of the Technique

It is now well established that phytoremediation can be cost-effective if aerial parts of plants are used as a raw material for the production of renewable energy (Sas-Nowosielska et al. 2004; Banuelos 2006; Robinson et al. 2009). On the other hand, the additional benefit to the cost ratio is unknown when bioaugmentation is associated with phytoextraction.

10.6 Methods for a Better Understanding of the Mechanisms Involved in Bioaugmentation-Phytoextraction Processes

10.6.1 Methods for Inoculant Monitoring, Microbial Biodiversity, and Microbial Activity

Traditional microbiological techniques often underestimate microbial numbers and do not allow the detection of noncultivable bacteria. Molecular techniques have

been developed to monitor inoculated microorganisms including the variations within microbial communities.

Various techniques can provide information on species composition of communities. Normalized extraction techniques should be used, since different DNA extraction procedures give differing results on fingerprinting (Crecchio et al. 2004). DNA hybridization techniques have been used to detect in situ microorganisms in environmental samples but may not be as sensitive as PCR techniques to monitor small communities; for example, real-time PCR allows for detection of less than 10^4 cells/g of soil (Rodrigues et al. 2002). RNA-based methods using 16S rRNA genes are more powerful than DNA-based one, since they allow monitoring metabolic active cells and gene expression. Genetic fingerprinting techniques characterize community variations and presence. A range of valuable techniques have been used, including T-RFLP, DGGE, TGGE, ARDRA, SSCP, DHPLC, ARISA, which can be coupled with sequencing (Widada et al. 2002). Among them, the most popular techniques are DGGE (denaturing gradient gel electrophoresis) and T-RFLP (terminal restriction fragment length polymorphism). Comparison of these two techniques shows that T-RFLP seems to be more sensitive than DGGE (Singh et al. 2006). Techniques to monitor microbial survival have been reviewed by Gentry et al. (2004). Unlike fingerprinting techniques, reporter genes are used to monitor inoculated bacteria and allow for distinguishing them from genetically close indigenous bacteria. These quantitative methods use mostly *gfp* and *lux* genes (Jansson 2003). Dual tagging techniques can be performed to detect both presence and activity of inoculated bacteria as shown by Unge et al. (1999), who monitored *P. fluorescens* SBW25 survival in soil after tagging with a *gfp-luxAB* cassette. Quantitative PCR (qPCR) and in situ PCR can be used to monitor specific gene expression of inoculated microorganisms, in situ PCR being less sensitive than qPCR for enumerating specific inoculated microorganisms (Tani et al. 2002). Fluorescence in situ hybridization (FISH) is also a powerful tool to detect microorganisms at different levels, such as group or individual species, and to give an indication of microbial presence and/or metabolic activity in situ (Lee et al. 2009). Cells can then be enumerated by epifluorescence microscopy or flow cytometry (Iwamoto and Nasu 2001). DNA microarrays have recently been developed to quantify bacterial DNA and to report gene expression in microbial communities. The main advantage of microarray technology is that expression of numerous genes can be monitored simultaneously; for example, the expression of 64 genes from *Ralstonia eutropha* JMP134 in mixed microbial communities was monitored with microarrays as a function of 2,4-D addition (Dennis et al. 2003). In complement with genomics, proteomics studies allow for the sorting of microbial responses to environmental conditions and for detection of key enzymes and molecules in bioaugmentation and bioremediation processes (Chauhan and Jain 2010). Moreover, communication between plants and bioaugmented microorganisms can be assessed by detecting and identifying proteins involved in their association, such as signal molecules. Proteomic studies have allowed, for example, the study of the PGPR effect of *P. fluorescens* on rice (Kandasamy et al. 2009).

10.6.2 Physicochemical and Biological Methods to Estimate Metal Bioavailability

Semple et al. (2004) estimate that until now, most routine chemical techniques typically estimate the bioaccessible fraction of metals, which must be taken into consideration as a matter of priority in bioremediation purposes. Estimating metal bioaccessibility depends on its form in the soil rather than on the total amount (Allen 1997; Zemberyova et al. 1998), and various analytical tools have been suggested. These include physical/chemical extraction techniques as well as an array of bioassays (Magrisso et al. 2008). Considerable research in environmental science has focused on chemical speciation of metals. It is increasingly realized that the distribution, mobility, and biological availability of chemical elements depend not simply on their concentrations, but on the chemical and physical associations that they undergo in natural systems (Zemberyova et al. 1998).

Two different approaches are usually applied in speciation studies for solid samples: single and sequential extraction procedures with reagents having different chemical properties. The chosen extractants simulate the influence of differing environmental conditions, such as acid rain, on the hypothetical release of metals from soil. Single-step extractions are more rapid and less expensive than sequential extractions, but they do not provide information on associations between metals and different soil fractions (Tack et al. 1996). For simple extraction in a single step, the extractants most commonly used are distilled water, DTPA, $NaNO_3$, or $CaCl_2$ (Adriano 1986; Gupta and Aten 1993; Van Ranst et al. 1999; Chen and Cutright 2001). A method developed by Feng et al. (2005) uses a mixture of acetic, lactic, citric, malic, and formic acids, which can mimic the action of root exudates on metal mobility.

Many schemes have been proposed for sequential extraction. In a review, Filgueiras et al. (2002) listed over 400 references published over the last decade relating to sequential extraction of metals. Some of these schemes may be variants of one another with minor variations in extractants and/or operating conditions. The results obtained with extraction procedures are, of course, operationally defined because redistribution and adsorption (Kheboian and Bauer 1987; Wang et al. 2003) usually take place during extraction. Possible oxidation of anoxic sediments due to the reagents used in the extraction process (Ngiam and Lim 2000) can be observed. The extraction efficiency is controlled by pH, concentration of extractant used, extraction time, temperature, and physicochemical properties of soil (McLaren 1998; Kalembkiewicz and Socco 2002). The particle size distribution and drying temperature, and therefore the presample processing, play an important role in metal speciation.

Chemical extractants tend to overestimate the concentration of phytoavailable metals, and correlation with shoot metal extraction is not always reliable; the effect was shown to be strongly dependent on the plant and metallic species (Gupta and Sinha 2006a, b). Regardless, however, the information obtained with these chemical methods is of great value to assess metal reactivity, and hence availability to the environment, and their potentially harmful effects (Abollino et al. 2002).

According to several studies, the free ionic metal concentration in the soil solution is a suitable indicator of what is extracted by plants corresponding to biodisponibility (Checkai et al. 1987, Csillag et al. 1999). Recently, a new technique called diffusive gradients in thin films (DGT) has been developed to assess heavy metal phytoavailability (Duquène et al. 2010; Pérez and Anderson 2009; Almas et al. 2006). This technique has shown a high positive correlation between DGT-extracted Cu and Cu concentration in *Lepidium heterophyllum* shoots (Zhang et al. 2001) but a low correlation level with Cu uptake by *Z. mays* (Cattani et al. 2006). Similarly, a poor correlation was demonstrated between DGT-measured U and U concentration in ryegrass shoots (Duquène et al. 2010).

The soil solution recovered by centrifugation is an alternate means to estimate this availability (Csillag et al. 1999; Zhang et al. 2001), requiring, however, a percentage of soil moisture above 80%. If chemical methods are not always relevant to measure bioavailable metal concentration, biological tools such as biosensors can be used. Significant correlations have been reported for Ni concentrations detected by the biosensor BIOMET, derived from *R. eutropha* CH34, and Ni accumulation by maize (Tibazarwa et al. 2001). Other biosensors have been created to assess bioavailable concentrations of Cr and Pb in soil with *R. metallidurans* AE 2440 (Corbisier et al. 1999) and Hg with a *mer-lux Escherichia coli* (Rasmussen et al. 2000). However, none of these biosensors has been used to predict metal uptake by plants.

10.7 Efficiency of Phytoextraction-Assisted Bioaugmentation

10.7.1 Evaluation of Phytoextraction Efficiency Must Incorporate Several Parameters

10.7.1.1 Plant Parameters

A minimum value of the metal concentration in aerial portions must be attained. The cost of biomass treatment decreases as metal concentration in the plant increases. Nonetheless, the key parameter for efficient phytoextraction is the amount of metal extracted by the aerial parts of plants and per surface area, taking into consideration both metal concentration and plant biomass. Accordingly, hyperaccumulating *T. caerulescens* whose Cd concentration in shoots was ten times higher than that recorded in *Salix* spp. extracted one-half Cd per hectare because of low biomass production (Hammer and Keller 2002). Because of their high biomass and extensive root system, trees are thus considered to be attractive for phytoextraction, although metal accumulation tends to be low (Glick 2010). Other parameters to evaluate phytoextraction efficiency include translocation (TF) and bioconcentration (BCF) factors (defined, respectively, as the ratio of metal concentration in shoots, metal concentration in soil and ratio of metals in shoots and roots), settling depth, ease of "mechanical harvesting, and the period and number of harvests expected per year based on phytoextraction rate (kinetic aspects).

10.7.1.2 Microbial Parameters

Microbial survival is an important parameter, but microbial activity appears more appropriate, since rate of metal extracted by plants and plant growth are not always shown to be correlated with inoculum size (Rai et al. 2004; Baum et al. 2006; Braud et al. 2006b). Root elongation, solubilization of insoluble phosphates, activity of enzymes such as ACC deaminase, and IAA production are also relevant parameters that contribute to estimation of bioaugmentation relevancy.

10.7.1.3 Efficiency of Phytoextraction-Assisted Bioaugmentation

In spite of the numerous experimental protocols and various means of exploiting results, some tendencies can be derived from the extensive data published on this topic (reviewed by Lebeau et al. 2008). What emerges is the following: (1) various metals and metalloids have been studied (As, Cd, Cr, Cu, Hg, Ni, Pb, Se, U, Zn); (2) multicontaminated soils and soils contaminated for long periods (not just prior to the experiments) each represented only 25% of the experiments, and most have been conducted with soils from industrial origin; (3) about 60% of soils were sterilized exacerbating the positive effect of bioaugmentation; (4) most experiments were performed in carefully controlled conditions (laboratories, greenhouse) and only a few in field conditions; and (5) microorganisms were selected from various environments such as the rhizosphere where microorganisms seem more tolerant to metals than in the bulk soil. Microbes were selected for their tolerance to metals more than for their ability to compete indigenous microorganisms and protozoa and in a few cases for their beneficial interaction with plants (growth and metal tolerance) and for their effect on the bioavailability of metals for plants.

The effect of microorganisms (especially PGPR and AMF) on plant biomass production and concentration of accumulated metal is shown in Table 10.2. The amount of metals extracted by shoots resulted in two main situations: (1) an increase (decrease) of plant biomass simultaneously to a decrease (increase) of metal concentration accumulated in plants. In such cases, bioaugmentation enhanced metal phytoextraction by a factor not exceeding 1.5 up to 2; and (2) the increase of both plant biomass and metal concentration as observed in a few studies and resulting in a fourfold higher metal amount in shoots (on average) with a maximum value that reached a factor of 34 (Di Gregorio et al. 2006). Most often, PGPRs

Table 10.2 Effect of soil bioaugmentation (compared to non-bioaugmented soils) on the increase in plant biomass and metal

		Root	Shoot
Plant biomass	Bacteria	1.1–2.6	1.2–4
	AMF	0.3–6.9	0.5–4.7
Plant metal concentration	Bacteria	1.2–5.6	1.1–3.1
	AMF	1.1–4.2	1.0–2.9

decrease TF, while AMF increases or decreases TF irrespective of metal species and concentration. Overall bioconcentration factor (BCF) was above unity with the highest values observed with less-contaminated soils.

10.8 Environmental Aspects

As with any in situ remediation process, the environmental impact of bioaugmentation-assisted phytoextraction must be evaluated. Indeed, one may be concerned that the increase of metal availability is greater than the plant accumulation ability (Barona et al. 2001) with a risk of contamination of subsoil and groundwater. This phenomenon has been observed for EDTA-assisted Pb extraction, resulting in more rapid mobilization of Pb by EDTA than its uptake rate by *Brassica rapa* L., *Vigna radiata* L., and *Triticum aestivum* L. (Shen et al. 2002; Chen et al. 2004b). Nonetheless, the same authors have shown that *Vetiver zizanioides* is able to recover all Cd, Cu, and Pb complexed with EDTA as a result of a well-developed root system.

Some bioindicators of the recovery of soil functioning following phytoextraction or bioaugmentation have already been used. Di Gregorio et al. (2006) have observed a severe modification of the bacterial community structure of the soil, using Denaturing Gradient Gel Electrophoresis (DGGE), due to cultivation with *B. juncea*. Conversely, EDTA only slightly affects bacterial community structure, with the exception of the simultaneous presence of *B. juncea* and PGPR *Sinorhizobium* sp. In contrast, pseudomonads inoculated in soils, either uncontaminated or contaminated with Cd, induced a shift in microbial communities, as suggested by analyzing in situ catabolic potential (Duponnois et al. 2006). Conversely, Epelde et al. (2009) showed a soil functioning restoration following Cd and Zn phytoextraction as reflected by the values of different microbial parameters. Metal phytoextraction level (hyperaccumulator vs. nonaccumulator plants) also affects microbial activity and community composition (Wang et al. 2008).

10.9 Future Prospects

During the past 10 years, microorganism-assisted phytoextraction has generated numerous experiments that have clearly shown the potential for increasing the quantity of metals extracted by plants (extensively reviewed by Lebeau et al. 2008). Although we now have a much better understanding of how microorganisms and plants interact in enhancing metal phytoextraction, one can wonder how great the concentration and amount of metals in plants can become. Some questions are still in abeyance, thus limiting the success of such a technique. For example, is the presence of inoculated microorganisms the limiting factor affecting metal bioaccessibility or the maximal amount of metals accumulated by plants? Low bioaccessibility of

metals in soil is often pointed out as the major limitation in phytoextraction efficiency (Pilon-Smits 2005). Soil bioaugmentation by siderophore-, biosurfactant-, or organic acid-producing microorganisms were shown to significantly enhance metal bioavailability (Braud et al. 2006b), siderophores being the most efficient. Nonetheless, Glick (2010) stated, in his review, that PGPR producing such molecules would only slightly modify metal accessibility in the presence of high concentrations of metals. To definitively conclude with that point, mass balances should be undertaken to estimate the surplus bioaccessible metal in the soil following bioaugmentation and to conclude whether the entirety is extracted by the plant or not. Braud et al. (2009b) showed that, in spite of a higher amount of Cr and Pb in both the bioaccessible fraction of the soil and in maize shoots following soil bioaugmentation, the amount of metals accumulated in the whole plant almost always decreased. This imbalance between metal supply and uptake was already observed when synthetic metal chelators were used (Shen et al. 2002; Chen et al. 2004a). Consequently, the concern persists that some metals will leave the root zone and generate an environmental risk that must be evaluated.

Regarding microbial–plant interactions, Audet and Charest (2007b) underscored a transitional role of AMF symbiosis in phytoremediation, shifting from "enhanced uptake" to "metal-binding" beyond critical metal levels. PGPRs seem less susceptible to metal load in the soil (Lebeau et al. 2008), and bioaugmentation could be performed at higher metal concentrations. Although some microbial activities were identified, i.e., IAA, siderophores, and ACC deaminase, in promoting plant growth, further research should unravel the contribution of each. In his review, Glick (2010) reported that the presence of all or even some of these activities could be sufficient to elaborate the entire mechanism regarding plant growth promotion. Using mutants not producing or overproducing these microbial activities will be very useful to better understand the mechanisms involved in plant growth and metal phytoextraction following bioaugmentation. An increased tolerance of *Medicago truncatula* was shown with IAA-overproducing strain *Sinorhizobium meliloti* DR-64 (Bianco and Defez 2009) as the result of proline accumulation and decreasing levels of the antioxidant enzyme superoxide dismutase, peroxidase, glutathione reductase, and ascorbate peroxidase. Some rhizosphere chemical dialogues reviewed by Badri et al. (2009) also occurred in the intricate microorganism–plant interactions. For example, a specific plant signal could be received by each rhizobacterium, making the microorganism-plant selection much more complex. Single host–single PGPR interactions are most often examined while multiple interactions exist in nature. The same authors stated that although abundant information is available on the role of root-secreted secondary metabolites in rhizospheric plant–microbe interactions, the role of exuded proteins is poorly studied.

There is also a need to clarify whether soil bioaugmentation effects on plant growth and metal phytoextraction are direct or indirect via indigenous microorganisms whose structure and activity could be altered by the supply of exogenous microorganisms.

Kinetic studies should also be performed to analyze both the length of time before plant harvest and the balance in the course of the time between surplus

bioaccessible metal following bioaugmentation, and metal uptake by plant. Indeed, it is never mentioned whether or not time of harvest corresponds to the maximal accumulation of metal in plants, while it is well known that nutrient needs vary greatly as does biomass during the growing period. For example, Cd concentrations in roots and leaves of the hydroponically cultivated *Allium schoenoprasum* increased until 7 days and were stable afterward until 28 days at the two concentrations of Cd tested while the biomass increased (Barazani et al. 2004). With *B. rapa* (Chinese cabbage), Ag, Al, and Si concentrations decreased over the course of the growing cycle, while B and Ca increased with crop age (Moreno et al. 2003). Regarding surplus bioaccessible metals following bioaugmentation, peak values of water-soluble Pb and Cd in soil solution were attained 48h and 72h, respectively, after soil inoculation with *Burkholderia* sp. J62 (Jiang et al. 2008). Similar results were attained after 1 week, at most, with *P. aeruginosa*, *P. fluorescens*, and *A. niger* for Cr and Pb (Braud et al. 2006b). The physiological state of the plant associated with the inoculant also plays an important role. With *B. juncea*, PGPR population size (and probably its activity) varies according to the growth stage of the plant: seedling stage > flowering stage > tillering stage (Wu et al. 2006a).

In the near future, the main question that arises will be the following: can we fully control in situ bioaugmentation-assisted phytoextraction, which is the primary goal of process engineering, in spite of the spatial and temporal variability of open ecosystems? According to Vogel and Walter (2001), microbial ecology issues (i.e., taking into account indigenous populations, environmental parameters together with phenotypic characteristics of the strains and procedures for inoculant introduction to determine activity, persistence, and performance of bioaugmented strains) are among the most important in bioaugmentation approaches, although, unfortunately, they are rarely addressed. The ecological engineering concept first defined in the 1960s (Odum 1962) should be taken into account in all experiments regarding microorganism-assisted metal phytoextraction. This implies the design, development, and maintenance of ecosystems by using engineering technologies based on ecological principles (Mitsch and Jørgensen 2004). Microbial communities are most probably the basis of microbial resource management in the domain of environmental biotechnology, according to Verstraete et al. (2007). The challenge for microbial ecologists is to develop the soil metabolome by introducing relevant microorganisms to benefit from their actions within the system.

To conclude, future prospects for in situ bioaugmentation technologies should be to reconcile process engineering, based on the full control of any system, with variable environmental conditions. Systems associating microorganisms and plants are relevant, since physicochemical conditions in the rhizosphere are less susceptible to change in the course of the remediation effort than in the bulk soil. Additionally, compared to the soil oligotrophy, rhizospheric soil contains a higher amount of nutrients regularly exuded by the plant (Gentry et al. 2004), ensuring a continuous substrate supply to microorganisms. Rhizosphere bioaugmentation could, thus, be considered a means to enhance both microbial survival and root colonization, as well as metal supplies to plants.

Experiments based on multifactorial designs are a prerequisite to unravel both the respective contribution of the main factors involved in metal phytoextraction and their interactions for a better understanding and optimization of microorganism-assisted phytoremediation. Unfortunately, only a few parameters are studied at a time, leading to a lack of some default values, thus precluding a full understanding of ecosystem functioning. The Taguchi method of design of experiments is a simple statistical tool that allows a maximum number of main effects to be estimated with a minimum number of experimental runs (Mohan et al. 2007; Rao et al. 2008). Proper design of experiments helps in gaining information about optimized levels, by addressing large numbers of variables over a specific region of interest. This approach concentrates on the effect of variation on process characteristics. The proposed method facilitates a systematic mathematical approach to understanding the complex bioremediation process and the optimization of near-optimum design parameters. For example, among eight biotic and abiotic factors (substrate loading rate, pH, dissolved O_2, soil/water ratio, temperature, soil microflora load, application of bioaugmentation, and humic substances concentration), substrate loading rate showed significant influence on bioremediation process (Mohan et al. 2007).

In open ecosystems, contrary to laboratory conditions, microorganisms are constantly exposed to multiple and continuously changing environmental stresses, preventing the information developed at one site from being used to design treatment strategies for other systems and pollution types. Therefore, predictions regarding microbial adaptive resiliency must be supplied by developing stress response systems as tools for effective and general process control (Hazen and Stahl 2006). Modeling is also a means of predicting bioremediation efficiency and to avoid too many experiments, but they are, however, unable to fully describe the huge complexity of ecosystems. Future prospects should consider the role of plants in bioremediation as a microbial regulating factor.

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Chapter 11
Biosorption of Uranium for Environmental Applications Using Bacteria Isolated from the Uranium Deposits

Takehiko Tsuruta

Abstract Attempts were made to recover uranium (U) occurring in nuclear fuel effluents and mine tailings using bacteria isolated from U deposits in Canada, the United States, Australia, and Japan. To establish which microorganisms accumulate the most U, hundreds of strains of microorganisms were screened. Extremely high U accumulating ability was detected in some bacteria isolated from North American U deposits. *Arthrobacter* and *Bacillus* sp. accumulated approx. 2,500 μmol U/g dry wt. of microbial cells within 1 h. Cells removed U from refining wastewater with high efficiency. Cells also accumulated thorium with high efficiency. *Lactobacillus* cells isolated from Japanese U deposits removed more U from seawater than the other bacteria that had superior U removal capacity from nonsaline U solutions. Cells immobilized with polyacrylamide gel had excellent handling characteristics and can be used repeatedly in U adsorption–desorption cycles. These bacteria from U deposits can be used as an adsorbing agent for the removal of the nuclear fuel elements, which may be present in nuclear effluents, mine tailings, seawater, and other waste sources.

11.1 Introduction

The recoveries of nuclear fuel elements, such as uranium (U) and thorium (Th), from aqueous systems have become a focus of interest for exploitation of undeveloped energy resources. The removal of radioactive elements and toxic heavy

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_11,

metals from contaminated sources is also a worthwhile priority for environmental protection initiatives. In this regard, efforts have concentrated on studying the accumulation of U by microorganisms, including bacteria (Andres et al. 1993; Byerley et al. 1987; Friiss and Myers-Keith 1986; Gorab et al. 1991; Hu et al. 1996; Marques et al. 1991; Strandberg et al. 1981), fungi (Byerley et al. 1987; Galun et al. 1983a, b; Tsezos and Volesky 1981; White and Gadds 1990), and yeasts (Strandberg et al. 1981; Shumate et al. 1978).

We have investigated U accumulation from aqueous systems using bacteria isolated from U mines, among which some strains were found to possess extremely high U accumulating ability (Sakaguchi et al. 1996). Microbial biomass may thus be considered for use as a removal agent for the recovery of U from metallurgical effluents, mine tailings, seawater, and other waste sources.

In U deposits, it can be presumed that some microorganisms having a high accumulating ability for U and different species having an ability to leach U from ore may exist in mine soil and aqueous systems. It would, therefore, be beneficial to isolate microorganisms having an enhanced ability to accumulate U from mines.

Recently, we screened hundreds of types of microorganisms existing in U deposits located in North America, Australia, and Japan for their ability to accumulate significant quantities of U, and identified new strains which accumulated large quantities of U, such as *Bacillus subtilis* in Australia, *Arthrobacter* and *Bacillus* sp. in North America, and *Lactobacillus* and *Bacillus* sp. in Japan (Sakaguchi 1998). In this chapter, new strains of bacteria identified in North American, Australian, and Japanese U deposits, especially *Arthrobacter*, *Lactobacter*, and *Bacillus* sp., are discussed for their potential for the removal of nuclear fuel elements such as U from U refining wastewater and seawater.

11.2 Screening of Microorganisms Isolated from U Deposits for Their U Accumulating Ability

To determine the ability of microorganisms isolated from U deposits in Canada, the United States, Australia, and Japan to accumulate U, hundreds of strains of microorganisms were screened.

The medium for growing microorganisms contained 3 g/L beef extract, 5 g/L peptone, and 5 g/L NaCl in deionized water. The microorganisms were maintained on agar slants and grown in 300 mL of the medium in a 500-mL flask with continuous shaking (120 rpm) for 72 h at 30°C. Cells were collected by centrifugation, washed thoroughly with deionized water, and then used in the following accumulation experiments.

U was supplied as $UO_2(NO_3)_2$. The pH of the solution was adjusted to 5.8 with 0.1 M HNO_3. Resting microorganisms (15 mg dry wt.) were suspended in 100 mL solution (pH 5.8) containing 84 μM U and the suspension was shaken for 1 h at 25°C. Cells were collected by filtration through a membrane filter (pore size 0.2 μm). The quantity of U accumulated by the cells was determined by measuring

U in the filtrate using an inductively coupled plasma quantometer (ICPS8000; Shimadzu Corporation, Kyoto, Japan). The microbial strains were identified by our coworker (Sakaguchi 1998).

The quantities of U accumulated by the cells ranged from a minimum of 10.9% to a maximum of 98.3% (Table 11.1). Of special interest to this discussion is the wide range of effectiveness with which different species of microorganisms accumulate U.

Of these microorganisms tested, extremely high U accumulating ability was found in *Arthrobacter* (96.4%) and *Bacillus* sp. (95.9%) found in US, *Lactobacillus* (97.8%) and *Bacillus* sp. (97.6%) found in Japan, and *Bacillus* sp. (98.3%) found in Australia (Sakaguchi 1998), which accumulated large quantities of U per gram dry wt. of microbial cells within 5 min.

11.2.1 Factors Affecting U Accumulation by Bacteria

In order to obtain basic information regarding the removal of U using strains of bacteria found in U deposits, some factors affecting U accumulation were investigated in detail using *Arthrobacter* sp., US-10 isolated from United States U deposits.

11.2.2 Effect of pH on U Accumulation

The pH of a test solution was adjusted to the desired value with 0.1 M HNO_3 or 0.1 M NaOH. Resting *Arthrobacter* cells (15 mg dry wt.) were suspended in 100 mL solution containing 84 μM U for 1 h at 25°C.

Additionally, Zeta potential was measured by the electrolysis method using 30–50 bacteria (bacterial concentration was 2.5×10^8 cells/mL) at pH 2–9 in 0.01 M $NaNO_3$ solution (ZC-2000; Microtec-Nichion, Chiba, Japan).

The effects of pH on U absorption using *Arthrobacter* sp., US-10 and *Lactobacillus* sp., JPN-10 are shown in Fig. 11.1. The amounts of absorbed U are highest at approx. pH 5–8 and decrease with increasing acidity below pH 4 using *Arthrobacter* cells. On the other hand, the amounts of absorbed U using *Lactobacillus* were highest at pH 6, and rapidly decreased below pH 5 and above pH 7. Low pH is the result, of course, of higher proton concentrations, which can compete for binding with U, and *Arthrobacter* cells clearly remained less affected than *Lactobacillus* by the decreasing pH until about pH 4. Conversely, the high pH is, of course, the result of reduced proton concentration and increased hydroxide ions, which compete with microbial cells. Again, *Arthrobacter* exhibited a higher pH range for binding capacity than *Lactobacillus*, achieving good absorption at pH values as high as 8, while *Lactobacillus* performed well only at pH 6.

The effects of pH on Zeta potential of the cell surface are shown in Table 11.2. The quantities of U accumulated are highest at approx. pH 5 and decrease below pH 4.

Table 11.1 Accumulation of uranium using microorganisms from soil or water at uranium deposit

Strain number	Uranium accumulated (%)	Strain number	Uranium accumulated (%)	Strain number	Uranium accumulated (%)	Strain number	Uranium accumulated (%)
US-1	14	CAN-1	12.6	AUS-1	19.4	JPN-1	10.9
US-2	17.1	CAN-2	23.7	AUS-2	25.4	JPN-2	21.2
US-3	23.9	CAN-3	33.9	AUS-3	36.6	JPN-3	32.2
US-4	38.7	CAN-4	49.8	AUS-4	46.2	JPN-4	44.7
US-5	43.9	CAN-5	55.7	AUS-5	53.3	JPN-5	54.9
US-6	58.9	CAN-6	67.9	AUS-6	68.8	JPN-6	65.2
US-7	72	CAN-7	74.2	AUS-7	74.7	JPN-7	76.8
US-8	84.4	CAN-8	85.7	AUS-8	85.1	JPN-8	86.2
Bacillus sp., US-9	95.9	CAN-9	95.4	AUS-9	96.9	*Bacillus* sp., JPN-9	97.6
Arthrobacter sp., US-10	96.4	CAN-10	96.9	*Bacillus* sp., AUS-10	98.3	*Lactobacillus* sp., JPN-10	97.8

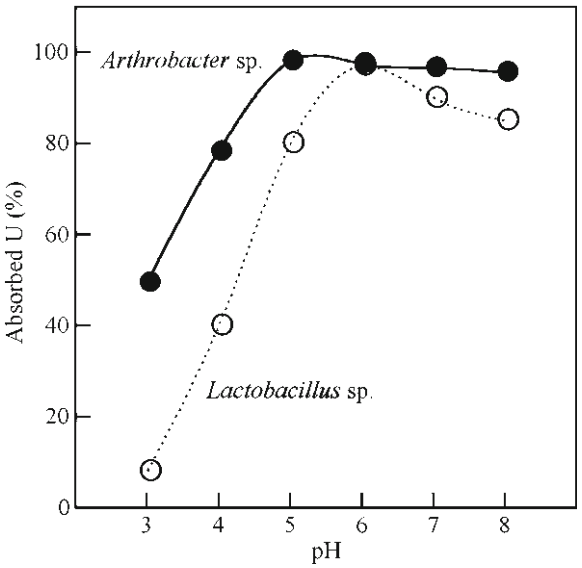

Fig. 11.1 Effects of pH on the amount of uranium absorbed using *Arthrobacter* and *Lactobacillus* cells. Symbols: *closed circles*, uranium (VI) accumulated (μmol/g dry wt. cells) using *Arthrobacter* cells; *open circles*, uranium (VI) absorbed (μmol/g dry wt. cells) using *Lactobacillus* cells

Table 11.2 Effect of pH on Zeta potential of *Arthrobacter* cell

pH	2.07	3.00	4.04	5.09	6.07	6.97	8.06
Zeta potential (mV)	3.69	−1.50	−7.33	−21.01	−23.18	−22.11	−23.06

Thus, the accumulation of U by *Arthrobacter* is markedly affected by solution pH. Zeta potential of the cell surface increased with increasing acidity of the solution.

The main U species at pH 6 includes some cations, such as $(UO_2)_3(OH)_5^+$ and UO_2OH^+ (Tsuruta 2006). On the other hand, Zeta potential of the cell surface was −23.18 mV at the same pH. Therefore, cationic uranyl species readily bond with negatively charged cell surfaces of *Arthrobacter* sp.

11.2.3 Effect of U Concentration on U Absorption

Resting *Arthrobacter* and *Lactobacillus* cells (15 mg dry wt.) were suspended in 200 mL of solution (pH 5.8) containing a specified quantity of U for 1 h at 25°C.

Quantities of absorbed U using *Arthrobacter* and *Lactobacillus* sp. (μmol U/g cells) increased as U concentration increased (Fig. 11.2). *Arthrobacter* and *Lactobacillus* cells accumulated 2,480 and 2,120 μmol U/g of cells, respectively.

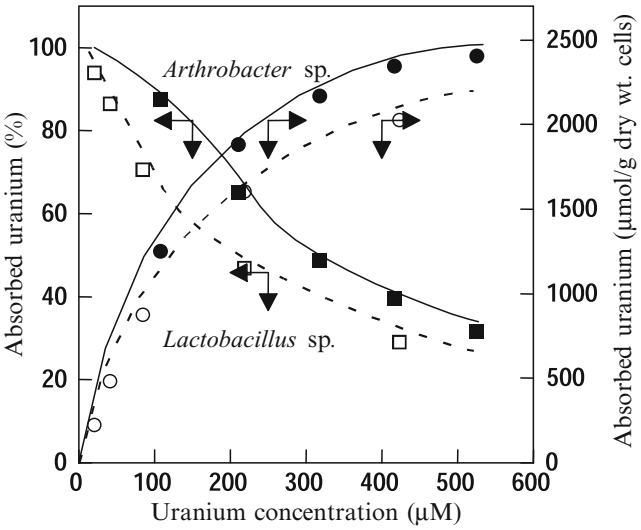

Fig. 11.2 Effect of uranium concentration on uranium absorption using *Arthrobacter*, US-10 and *Lactobacillus*, JPN-10 cells. Symbols: *circles*, accumulated uranium (μmol/g dry wt. cells); *squares*, accumulated uranium (%); *closed*, absorbed uranium using *Arthrobacter* sp.; *open*, absorbed uranium using *Lactobacillus* sp.

Additionally, U absorption using these cells, especially *Lactobacillus* sp., does not obey the *Langmuir* isotherm over the entire U concentration range tested. It appears that the experimental data show a dual pattern. The dotted line was calculated using less than a 21.8-μM residual U concentration (as the initial uranium concentration was 85.0 μM) using *Lactobacillus* cells. The solid line was separately calculated using residual concentrations below and above 21.8 μM. When the initial U concentration increased beyond 85.0 μM (with a 21.8-μM residual U concentration), the absorbed quantity increased to values far greater than those calculated based on the relationship in the low concentration range. On the other hand, the dotted line in Fig. 11.3 for *Arthrobacter* cells was calculated using a residual U concentration below 153 μM (and an initial U concentration of 318 μM). The solid line was separately calculated for *Arthrobacter* cells using residual concentrations below and above 153 μM. When the initial U concentration increased beyond 318 μM (with a residual U concentration of 153 μM), the absorbed quantity increased to values a little greater than those calculated based on the relationship in the low concentration range. A similar result was obtained (Epstein 1966) from the absorption of potassium using barley roots. The estimated m, n, and maximum accumulated U capacity $Q(\mathrm{U})_{\max}$ (=1/m) are summarized in Table 11.3. A high $Q(\mathrm{U})_{\max}$ value of 2,580 and 2,370 μmol U/g dry wt. cells is estimated from the high U concentration region using *Arthrobacter* and *Lactobacillus* cells, respectively.

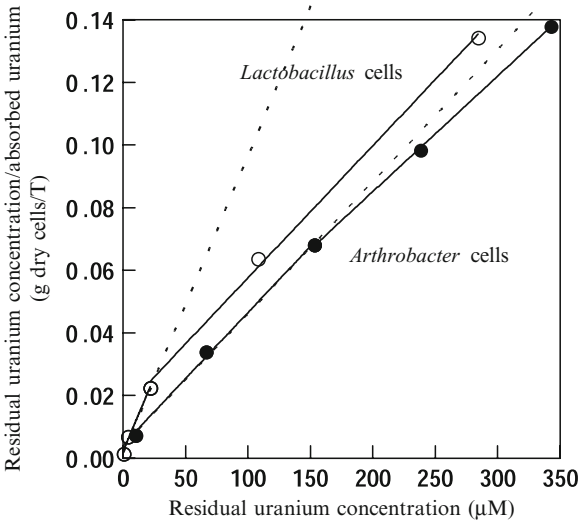

Fig. 11.3 Equilibrium isotherm of uranium absorption using microbial cells. Symbols: *closed*, absorbed uranium using *Arthrobacter* sp.; *open*, absorbed uranium using *Lactobacillus* sp. $C_e(U)/Q_U = mC_e(U) + n$, where Q_U indicates the amount of absorbed uranium (μmol uranium/g dry wt. cells), C_e is the residual uranium in the solution (μM), and m and n are constants

Table 11.3 Estimated Langmuir constants and $Q(U)_{max}$ from the Langmuir isotherm

Strains	Initial uranium concentration (μM)	Residual uranium concentration (μM)	m	n	$Q(U)_{max}$ (μmol/g dry cells)
Arthrobacter sp.	107–318	10.0–153	4.22×10^{-4}	4.32×10^{-3}	2,371
	318–525	153–343	3.69×10^{-4}	1.13×10^{-2}	2,710
	107–525	10.0–343	3.88×10^{-4}	6.45×10^{-3}	2,580
Lactobacillus sp.	21.0–85.0	0.483–21.8	9.51×10^{-4}	2.05×10^{-3}	1,050
	85.0–423	21.8–284	4.22×10^{-4}	1.53×10^{-2}	2,370
	21.0–423	0–284	4.56×10^{-4}	769×10^{-3}	2,200

See the legend of Fig. 11.3

11.2.4 Time Course of U Accumulation

Resting *Arthrobacter* and *Lactobacillus* cells (15 mg dry wt.) were suspended in 100 mL solution (pH 5.8) containing 84 μM U at 25°C.

Quantities of U accumulated by *Arthrobacter* and *Lactobacillus* cells increased rapidly during the first 5 min following the application of U (Fig. 11.4).

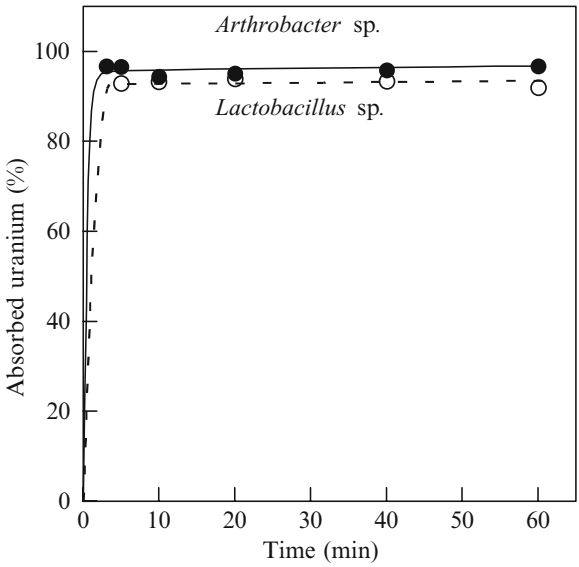

Fig. 11.4 Time course of uranium absorption using *Arthrobacter* and *Lactobacillus* cells. Symbols: *closed*, absorbed uranium using *Arthrobacter* sp.; *open*, absorbed uranium using *Lactobacillus* sp.

11.2.5 Release of U from Cells by Washing with EDTA

Resting cells (15 mg dry wt.) were suspended in 100 mL solution (pH 6.0) containing 84 μM uranium for 1 h at 25°C. Cells with accumulated U were washed three times with 10 mL of 10 mM EDTA solution.

When cells of *Arthrobacter* sp., US-10, *Bacillus* sp., US-9; and *Lactobacillus* sp., JPN-10 were washed with EDTA, approx. 70, 80, and 53% of the accumulated U was desorbed from the resting cells, suggesting that most U is coupled with ligands that are easily substituted by EDTA (Fig. 11.5). However, 30, 20, and 47% of accumulated U in *Arthrobacter*, *Bacillus*, and *Lactobacillus* sp. were therefore not substituted by EDTA washing that incorporated within cell membranes. The ratio of released U (%) occurred in the following order: *Bacillus* sp.>*Arthrobacter* sp.>*Lactobacillus* sp.

11.2.6 Distribution of U in Microbial Cells

The present experiments were undertaken to determine which parts of the cells had accumulated U in *Arthrobacter*, US-10 and *Lactobacillus*, JPN-10 cells.

Resting cells of *Arthrobacter* and *Lactobacillus* (640 mg fresh weight) were suspended in 1,000 mL solution (pH 5.8) containing 500 μM of U for 1 h at 25°C. The cells were fractionated as described in Fig. 11.6. The freeze-dried *Arthrobacter*

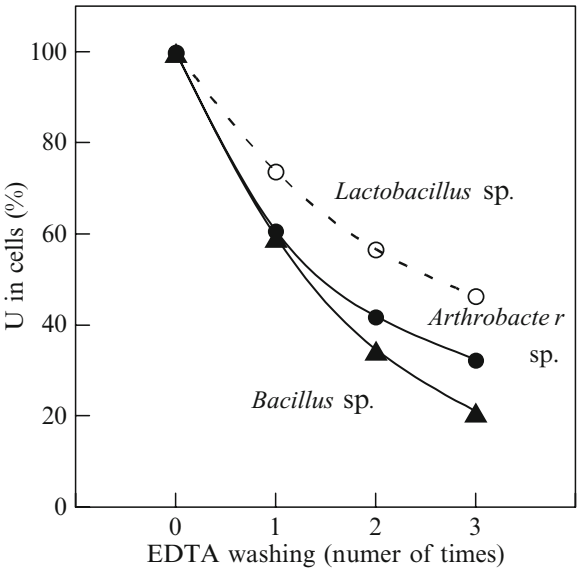

Fig. 11.5 Release of uranium from *Lactobacillus* sp., JPN-10; *Arthrobacter* sp., US-10; or *Bacillus* sp., US-9 cells by washing with EDTA

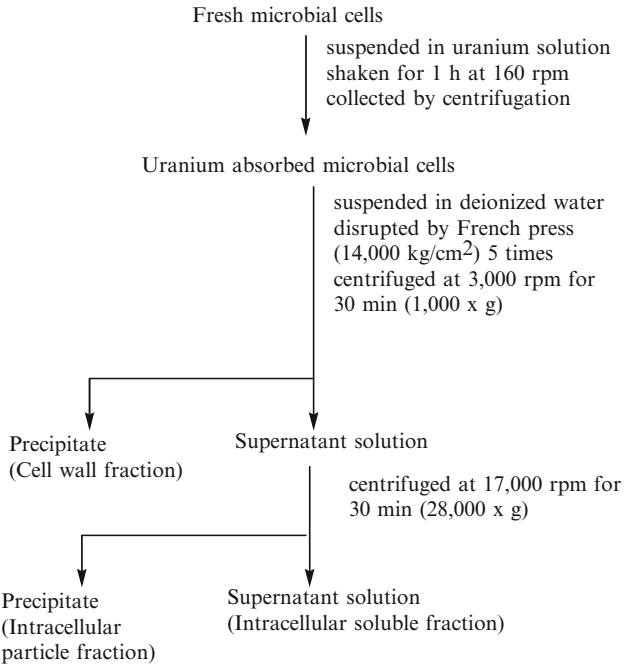

Fig. 11.6 Fractionation of *Lactobacillus* sp., JPN-10, *Arthrobacter* sp., US-10 absorbed uranium

Table 11.4 Distribution of uranium in microbial cells

	Absorbed uranium			
	Arthrobacter sp.		*Lactobacillus* sp.	
Fractions	(μmol)	(%)	(μmol)	(%)
Whole cells	237	100	220	100
Cell wall fraction	140	59	209	95
Intracellular particle fraction	51	21	11	5
Intracellular soluble fraction	32	13	4	2

and *Lactobacillus* cells, cell wall, and intracellular particle fractions were digested in the mixed solution of same volume of conc. HNO_3/H_2SO_4.

The quantities of accumulated U in each fraction were recorded in the following order (Table 11.4)

Cell wall > intracellular particle > intracellular soluble.

Abundant U was determined in the cell wall fraction and small amounts occurred in the intracellular fractions. In the case of *Arthrobacter*, this result coincides with the results noted above for EDTA washing. On the other hand, the results using *Lactobacillus* suggested that most accumulated U was coupled with the cell wall fraction; half of the accumulated U was not released by washing with EDTA. Therefore, the bond of U with cell wall of *Lactobacillus* cells appears to be strong.

On the basis of these findings, it seems reasonable to postulate that the accumulated U using the cells, especially *Lactobacillus* sp. is mostly dependent on the physical–chemical binding relationships with cell wall components.

11.2.7 Selective Accumulation of U Using Arthrobacter, US-10 Cells

To determine which heavy metal ions are most readily accumulated by bacterial cells, the selective accumulation of ions using *Arthrobacter*, US-10 cells from a solution containing six metal cations and UO_2^{2+} was examined. Resting cells (15 mg dry wt.) were suspended in 100 mL of a solution (pH 5.0) containing 4×10^{-5} M of Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and UO_2^{2+} for 1 h at 25°C.

The relative degree of heavy metal ions accumulated using *Arthrobacter*, US-10 cells was (Fig. 11.7) $UO_2^{2+} > Cu^{2+} >$ others.

11.3 Accumulation of Th and Selective Accumulation of Th and U by Bacteria

As described above, bacteria found in U deposits accumulated U with high efficiency. In this course of our study, the question was raised as to whether these strains had the ability to accumulate Th, another common waste from nuclear processing (and an environmental contaminant), as well as U.

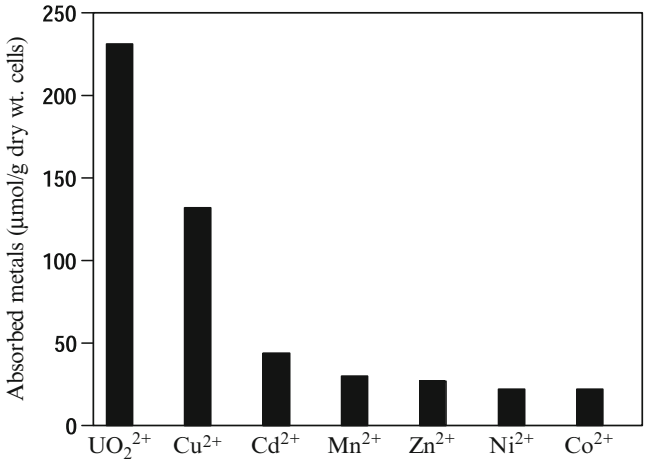

Fig. 11.7 Selective accumulation of heavy metals using *Arthrobacter*, US-10 cells

Table 11.5 Accumulation of thorium and/or uranium from the solution containing thorium and/or uranium

	Metal accumulated from the solution containing Th or U only (μmol/g dry wt. cells)		Metals accumulated from the mixed solution containing Th and U (μmol/g dry wt. cells)	
Strains	Th	U	Th	U
Arthrobacter sp.	98.8	98.9	98.5	52.1
Bacillus sp.	94.0	99.7	98.3	15.8
Lactobacillus sp.	47.1	64.9	46.7	17.9

As thorium hydroxide is precipitated in a solution containing thorium at pH 4.0, the accumulation of Th is examined at pH 3.5. Resting cells (15 mg dry wt.) were suspended in 100 mL solution (pH 3.5) containing 50 μM Th (as $Th(NO_3)_4$) and/or U for 1 h at 25°C.

Both *Arthrobacter* sp., US-10 and *Bacillus* sp., US-9 can also accumulate Th with high efficiency. However, the quantities of U and Th accumulated by *Lactobacillus* from the solution containing one metal only were lower than those using *Arthrobacter* and *Bacillus* sp. These results appear reasonable, because solution pH strongly affects the accumulation of both elements using *Lactobacillus* cells. The quality of accumulated U from the solution containing both elements at pH 3.5 by *Arthrobacter* was half that from the solution containing U only; however, that accumulated by *Bacillus* from the solution containing both elements was far lower than that from the U-only solution. Accordingly, the effect of Th on U accumulation by *Bacillus* is greater than that by *Arthrobacter*. Thus, the *Arthrobacter* sp. appears to be the most efficient choice for a mixed solution of Th and U (Table 11.5).

11.3.1 Recovery of U by Immobilized Bacteria

As described above, bacteria such as *Arthrobacter*, *Bacillus*, and *Lactobacillus* sp. can accumulate large quantities of U from aqueous systems. However, the free cells of these bacteria are not reusable because of their mechanical instability and susceptibility to cell degradation. Furthermore, free cells are not suitable for use in column systems, because they cause plugging. To overcome these deficiencies with free cells, the cells of *Arthrobacter* sp., US-10 having high U accumulating ability were immobilized with polyacrylamide.

Five grams of precultured *Arthrobacter* cells were suspended in 4.5 mL isotonic NaCl solution and 680 mg acrylamide monomer. A total of 34 mg *N*,*N*′-methylenebis(acrylamide), 0.3 mL 3-dimethylaminopropionitrile solution (5%), and 0.34 mL potassium persulfate solution (2.5%) were added to the suspension. After solidification, the gel was crushed into small pieces (50–100 mesh), washed thoroughly with isotonic NaCl solution followed by deionized water, and then used for adsorption experiments.

To obtain basic information on the recovery of U using immobilized microbial cells, U adsorption–desorption cycle tests were carried out. It was previously shown (Sakaguchi et al. 1996) that the U retained on the adsorbent can easily be desorbed with dilute Na_2CO_3 solution, so 0.1 M Na_2CO_3 solution was used as the desorbent in this experiment.

Fifteen milliliters of a solution (pH 5.8) containing 42 μM U was adsorbed on a column (bed volume, 2 mL) of immobilized *Arthrobacter* cells at a space velocity of 20 h^{-1}. Adsorbed uranium was desorbed with 10 mL of 0.1 M Na_2CO_3 solution. The test was replicated five times.

The ability of the immobilized *Arthrobacter* cells to adsorb U did not decrease after six repetitions of adsorption–desorption cycles (Fig. 11.8). Thus, immobilized microbial cells appear to have excellent handling characteristics and can be used repeatedly in adsorption–desorption cycles.

11.3.2 Removal of U from U Refining Wastewater by Bacteria

As mentioned above, some microbial species have a high U accumulating ability, which suggests the possibility that they may be used for the removal of U from U mine tailings, U refining wastewater, and other waste sources.

We attempted to remove U from U refining wastewater sampled at the Ningyotoge Environmental Engineering Center of the Japan Atomic Energy Agency using bacteria exhibiting a significant ability to accumulate U. Resting cells (15.0 mg dry wt.) were suspended in 100 mL of a solution (pH 6.0) of wastewater containing 21.0 μM U for 1 h at 25°C.

Lactobacillus and *Bacillus* sp. isolated from Japanese U deposits removed 88.1 and 74.4% U, respectively (Table 11.6), when solution pH was adjusted initially to

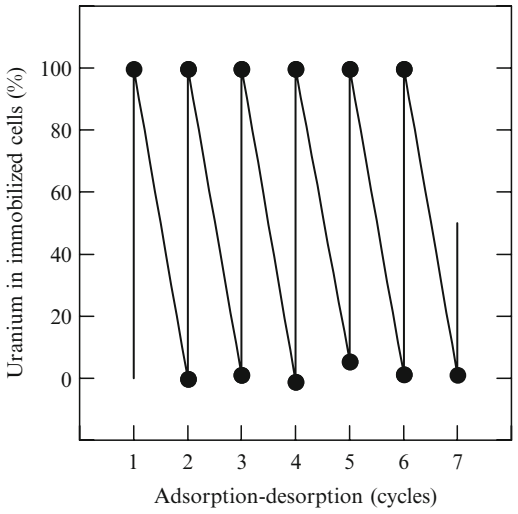

Fig. 11.8 Test of repeated uranium adsorption–desorption using immobilized *Arthrobacter*, US-10 cells

Table 11.6 Uranium removal from uranium refining wastewater using microbial cells isolated from Japanese uranium mine

	Removed U (%)	
Strains	pH adjusted only started at pH 6.0	pH adjusted continuously at pH 6.0
Lactobacillus sp.	88.1	99.5
Bacillus sp.	74.4	95.5

Table 11.7 Uranium removal from uranium refining wastewater using immobilized microorganisms isolated from uranium mines

Strains	Adsorbed uranium U (%)
Arthrobacter sp.	100
Bacillus sp.	100

6.0. Solution pH gradually decreased, with *Bacillus* cells being more adversely affected by pH change than *Lactobacillus* cells. However, both strains quantitatively removed U when the pH was maintained at 6.0. These species can thus remove U from U refining wastewater with a high efficiency.

Attempts were also made to remove U from U refining wastewater using immobilized microorganisms having a high ability to adsorb U. Uranium refining wastewater (100 mL, pH 6.0) supplemented with 2.1 mM of U were adsorbed on a column (bed volume 2 mL) of immobilized bacterial cells at a space velocity of 10 h^{-1} at 25°C.

Immobilized bacterial cells isolated from U mines in the United States can also remove U from the U refining wastewater with high efficiency (Table 11.7).

Table 11.8 Accumulation of uranium using microorganisms isolated from uranium mines

Solutions	Accumulated U (%)		
	Lactobacillus sp.	*Arthrobacter* sp.	*Bacillus* sp.
Uranium only solution (pH 8)	94.7	94.2	94.6
Natural seawater	36.2	0.8	0.9
Decarbonated seawater	70.2	6.1	6.0

11.3.3 Removal of U from Seawater by Bacteria

The removal of U from seawater supplemented with 4.2 μM U using the bacteria isolated from U deposits was examined. The concentration of carbonate in seawater is ~2.34×10^{-3} M (Ogata et al. 1971). The amount of U removed by *Chlorella* cells from solutions containing 1.196×10^{-3} M sodium hydrogen carbonate was less at pH values above 6 than at pH 5 (Nakajima et al. 1979). The decrease in the amount of removed U from solutions containing carbonate was estimated from the amount of the UO_2CO_3 formed at pH 6 and of $UO_2(CO_3)_3^{4-}$ formed at pH values greater than 7 (Nakajima et al. 1979, 1981). Although *Lactobacillus* sp. removed 36.2% of U from seawater, it removed nearly twice as much (70.2%) when the seawater was decarbonated (Table 11.8). *Arthrobacter* and *Bacillus* cells, which can remove large amounts of U from nonsaline water, removed far less U from either seawater or decarbonated seawater than did *Lactobacillus*. Accordingly, *Lactobacillus* has great potential in applications to remove significant quantities of U from seawater.

11.4 Conclusion

In U deposits located in Canada, the United States, Australia, and Japan, we isolated strains of bacteria such as *Arthrobacter*, *Bacillus*, and *Lactobacillus* sp. having a significant ability to accumulate U. These species could accumulate approx. 2,500 μmol U/g dry wt. of microbial cells within 1 h. These strains accumulated U selectively from solution containing six other heavy metals in solution. Cells also accumulated Th as well as U with high efficiency. These species removed U from U refining wastewater with high efficiency. *Lactobacillus* also accumulated U from seawater more effectively than other microbial cells which have high accumulating capacities, from nonsaline U solution.

Cells immobilized with polyacrylamide gel have excellent handling characteristics and can be used repeatedly in U adsorption–desorption cycles.

These strains of *Arthrobacter*, *Bacillus*, and *Lactobacillus* can be used as an adsorbing agent for the removal of nuclear fuel elements which may be present in nuclear fuel processing effluents, mine tailings, seawater, and other environmental sources.

Acknowledgements I thank professor Tsuyoshi Hirajima, Keiko Sasaki, and Mr. Yuki Aiba of the Department of Earth Resources Engineering, Faculty of Engineering, Kyushu University, for their measurement of Zeta potentials.

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Chapter 12
Bacterial Biosorption: A Technique for Remediation of Heavy Metals

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Abstract Bacterial biosorption can be used for the removal of pollutants from waters contaminated with pollutants that are not easily biodegradable, such as metals and dyes. A variety of biomaterials are known to bind these pollutants including bacteria, fungi, algae, and certain industrial and agricultural wastes. Biosorbents are less costly and more effective alternatives for the removal of metallic elements, especially heavy metals, from aqueous solution. In this chapter, the sorption abilities of bacterial biomass toward metal ions are emphasized. The appropriate conditions for immobilizing bacteria for maximum biosorption and the mechanism(s) involved are highlighted. The properties of cell wall constituents, such as peptidoglycan, and the role of functional groups, such as carboxyl, amine, and phosphonate, are discussed on the basis of their biosorption potentials. Binding mechanisms as well as the parameters influencing passive uptake of pollutants are analyzed. A detailed description of isotherm and kinetic models and the importance of mechanistic modeling are presented. To enhance biosorption capacity, biomass modifications through chemical methods and genetic engineering are needed for the effective removal of metal. For continuous treatment of effluents, a packed column configuration is suggested and the factors influencing its performance are discussed. The chapter also highlights the necessity for examination of biosorbents within real-world situations, as competition between solutes and water quality may affect biosorption performance. Thus, this chapter reviews the achievements and current status of biosorption technology and provides insights into this research frontier.

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_12,

12.1 Introduction

Enormous quantities of toxic metals are released into the environment annually as a result of human activities. In some cases, these releases are deliberate and well-regulated, like industrial emissions, while in other cases they are accidental and include chemical spills or improper land disposal (Lloyd 2002). Toxic metals of concern include lead, chromium, mercury, uranium, selenium, zinc, arsenic, cadmium, gold, silver, copper, and nickel. These pollutants are derived from mining, metallurgical, electronic, electroplating, chrome tanning, textiles, metal finishing, fertilizer manufacture, and steel and automobile industries. Quantities of heavy metals released into the environment have increased due to rapid industrialization and technological development, posing significant threats to ecosystems and public health because of their toxicity, accumulation in food chains, and persistence in nature (Sharma et al. 2006; Tuzen et al. 2008). Following the fate of toxic metal species after they enter the ecosystem becomes difficult; furthermore, they inflict damage as they move from one ecological trophic layer to another. Controlling heavy metal discharges and removing toxic heavy metals from water bodies has become a challenge for the twenty-first century.

Methods used for heavy metal removal from industrial effluents can be classified as physical, chemical, and biological. Physicochemical methods such as precipitation, ion exchange, filtration, membrane and electrochemical technologies, reverse osmosis, electrodialysis, adsorption on activated carbon, etc. require high capital and operating costs and may also be associated with the generation of secondary wastes which cause treatment problems. Therefore, recent attention has been drawn toward the development of alternative methodologies known as bioremediation processes. These technologies include, among other processes, biosorption. Biosorption or bioadsorption involves passive immobilization of metals by living biomass. Biosorption can be defined as the ability of biological materials to accumulate heavy metals from wastewaters through metabolically mediated or physiochemical pathways of uptake. Biosorbents for the removal of metals mainly come under the following categories: bacteria, fungi, algae, industrial wastes, agricultural wastes, and various polysaccharide materials. These biosorbents can effectively sequester dissolved metal ions from dilute complex solutions. The use of biological material in the removal of heavy metals from industrial effluents has gained importance during recent years because of the high efficiency, minimization of chemical/biological sludge, low operating cost, regeneration of biosorbents, and possibility of metal recovery.

12.2 Bacterial Biosorbents

Bacteria are the most abundant and versatile of microorganisms and constitute a significant fraction of the entire living terrestrial biomass, whose mass is estimated as ~10^{18} g (Mann 1990). In the early 1980s, certain microorganisms were found to

accumulate metallic elements at a high capacity (Vijayaraghavan and Yun 2008a, b). Biosorbents derived from bacterial biomass have since become popular because of their small size, ability to grow under controlled conditions, and their resilience to a wide range of environmental situations; furthermore, inexpensive nutrient sources are readily available for microbes. Potent metal biosorbents among bacteria include genera *Bacillus*, *Pseudomonas*, *Streptomyces*, *Micrococcus*, and *Escherichia coli*. Table 12.1 summarizes basic information regarding the use of bacterial biomass for metal biosorption.

Metal ions in solution are adsorbed on to bacterial surfaces through interactions with chemical functional groups such as carboxylate, amine, amide, imidazole, phosphate, thioether, hydroxyl, and other functional groups found in cell wall biopolymers. Biosorption includes a combination of several mechanisms such as electrostatic attraction, complexation, ion exchange, covalent binding, van der Waal's forces, adsorption, and microprecipitation. The extent of biosorption not only depends on the type of metal ions, but also on the bacterial genus, due to variations in cellular constituents. Very short contact times are generally sufficient to attain a metal-bacterial biomass steady state. This is because biomass is used in the form of either fine powder or wet cells, where mass transfer resistances are usually negligible. The rapid kinetics observed with bacterial biomass represents an advantageous aspect for the design of wastewater treatment systems.

12.2.1 Bacterial Structure

The diameter of typical bacterial cells range from 0.5 to 1.0 μm; however, some are wider than 50 μm. Bacteria have simple morphology; the most common bacteria are present in three basic shapes: spherical or ovoid (coccus), rod (bacillus, with a cylindrical shape), and spiral (spirillum), although there is a great variety of shapes due to differences in genetics and ecology. The small size of bacteria ensures rapid metabolic processes. A "typical" bacterial cell (e.g., *E. coli*) contains a cell wall, cell membrane, and the cytoplasmic matrix which consists of several constituents that are not membrane enclosed (inclusion bodies, ribosomes, and the nucleoid with its genetic material). Bacteria are classified as either Gram-positive or Gram-negative as distinguished by the Gram stain (Beveridge 2001). This classification divides bacteria into two main groups that differ in their cell wall characteristics (Beveridge 1989; Sleytr and Beveridge 1999). Both cell wall types encompass a peptidoglycan layer that is rich in carboxylate groups and completely surrounds the cell (Beveridge 1989; Langley and Beveridge 1999). The peptidoglycan layer in the Gram-positive cell wall is ca. 25 nm thick, whereas the Gram-negative peptidoglycan layer is much thinner (ca. 7.5 nm). The walls of Gram-positive bacteria consist of three primary components: cytoplasm mixed with peptidoglycan, to which teichoic acids are covalently bound. The envelope of Gram-negative bacteria is more complex than that of Gram-positive bacteria. It consists of two membrane bilayers (the outer and plasma membrane) that are chemically and functionally distinct from one another and sandwich a thin peptidoglycan layer between them. Teichoic acids give the Gram-positive

Table 12.1 Biosorption by bacterial biomass (mg g^{-1})

Metal	Bacteria species	pH	Biosorption capacity (mg g^{-1})	References
Cr (III)	*Pseudomonas aeruginosa* AT18	7.72	200	Silva et al. (2009)
	Rhodococcus opacus	6	1.404	Bueno et al. (2008)
	R. opacus	5	714.29	Torem et al. (2008)
	Staphylococcus saprophyticus BMSZ71	5	22.06	Zamil et al. (2009)
Cr (VI)	*Aeromonas caviae*	2.5	284.4	Loukidou et al. (2004)
	Arthrobacter sp.	4	9.115	Mishra and Doble (2008)
	Arthrobacter sp.	5	175.87	Hasan and Srivastava (2009)
	Bacillus licheniformis	2.5	69.4	Zhou et al. (2007)
	Bacillus marisflavi	4	5.783	Mishra and Doble (2008)
	Bacillus thuringiensis	2	83.3	Şahin and Öztürk (2005)
	Chryseomonas luteola	4	3	Ozdemir and Baysal (2004)
	Escherichia coli	4.6–5.1	4.6	Quintelas et al. (2009)
	Pseudomonas sp.	4	95	Ziagova et al. (2007)
	P. aeruginosa	NA	0.05	Kang et al. (2007)
	Pseudomonas fluorescence TEM08	2	40.8	Uzel and Ozdemir (2009)
	Staphylococcus sp.	1	143	Ziagova et al. (2007)
Cu(II)	*Arthrobacter* sp.	5	175.87	Hasan and Srivastava (2009)
	Bacillus sp. F19	4.8	89.62	Yan et al. (2008)
	Bacillus cereus	NA	50.32	Jian-hua et al. (2007)
	Geobacillus toebii	4	48.5	Ozdemir et al. (2009)
	Geobacillus thermoleovorans	4	41.5	Ozdemir et al. (2009)
	Enterobacter sp. J1	5	32.5	Lu et al. (2006)
	Pseudomonas sp.	8	0.046	Choudhary and Sar (2009)
	P. aeruginosa AT18	6.25	86.95	Silva et al. (2009)
	R. opacus	6	0.506	Bueno et al. (2008)
	Shewanella putrefaciens	NA	45	Chubar et al. (2008)
	Sphaerotilus natans	6	60	Beolchini et al. (2006)
	Streptomyces coelicolor	5	66.7	Öztürk et al. (2004)
	S. saprophyticus BMSZ71	6	22.36	Zamil et al. (2009)
	Thiobacillus ferroxidans	6	198.5	Liu et al. (2004)
Cd (II)	*A. caviae*	7	155.3	Loukidou et al. (2004)
	Bacillus circulans	7	26.5	Yilmaz and Ensari (2005)
	Bacillus jeotgali	7	57.9	Green-Ruiz et al. (2008)
	G. toebii	6	29.2	Ozdemir et al. (2009)
	G. thermoleovorans	4	38.8	Ozdemir et al. (2009)
	Enterobacter sp. J1	6	46.2	Lu et al. (2006)
	E. coli	5	2.18	Kao et al. (2009)
	E. coli	5.6–6	10.3	Quintelas et al. (2009)
	Pseudomonas sp.	7	278	Ziagova et al. (2007)
	Pseudomonas veronii 2E	7.5	54	Vullo et al. (2008)
	Pseudomonas sp.	9	0.078	Choudhary and Sar (2009)

(continued)

Table 12.1 (continued)

Metal	Bacteria species	pH	Biosorption capacity ($mg\,g^{-1}$)	References
	Streptomyces rimosus	8	64.9	Selatnia et al. (2004a)
	S. saprophyticus BMSZ71	7	54.91	Zamil et al. (2009)
Fe (II)	*E. coli*	2.7–3.5	16.5	Quintelas et al. (2009)
	S. rimosus	NA	122	Selatnia et al. (2004b)
Hg (II)	*Bacillus* sp.	6	7.9	Green-Ruiz (2006)
	S. saprophyticus BMSZ71	6	78.17	Zamil et al. (2009)
Ni (II)	*B. thuringiensis*	6	45.9	Öztürk (2007)
	E. coli	5.7–6.2	6.9	Quintelas et al. (2009)
	G. toebii	4	21	Ozdemir et al. (2009)
	G. thermoleovorans	4	42	Ozdemir et al. (2009)
	Pseudomonas sp.	8	0.062	Choudhary and Sar (2009)
	P. aeruginosa ASU6a	6	70	Gabr et al. (2008)
	P. fluorescence TEM08	2	40.8	Uzel and Ozdemir (2009)
	R. opacus	5	7.63	Cayllahua et al. (2009)
	S. saprophyticus	7	16.85	Zamil et al. (2009)
	S. coelicolor	11.1	8	Öztürk et al. (2004)
Pd	*Desulfovibrio desulfuricans*	2	128.2	de Vargas et al. (2004)
	Desulfovibrio fructosivorans	2	119.8	de Vargas et al. (2004)
	Desulfovibrio vulgaris	2	106.3	de Vargas et al. (2004)
Pt	*D. desulfuricans*	2	62.5	de Vargas et al. (2004)
	D. fructosivorans	2	32.3	de Vargas et al. (2004)
	D. vulgaris	2	40.1	de Vargas et al. (2004)
Zn	*B. jeotgali*	7	222.2	Green-Ruiz et al. (2008)
	G. toebii	5	21.1	Ozdemir et al. (2009)
	G. thermoleovorans	4	29	Ozdemir et al. (2009)
	P. aeruginosa AT18	7.72	56.4	Silva et al. (2009)
	Pseudomonas putida	5	17.7	Chen et al. (2005)
	S. putrefaciens	NA	22	Chubar et al. (2008)
	S. saprophyticus BMSZ71	26.33	7	Zamil et al. (2009)
	T. ferroxidans	6	82.6	Liu et al. (2004)
U	*Arthobacter nicotianae*	3.5	68.8	Nakajima and Tsuruta (2004)
	B. licheniformis	3.5	45.9	Nakajima and Tsuruta (2004)
	Bacillus megaterium	3.5	37.8	Nakajima and Tsuruta (2004)
	Bacillus polymyxa IMV 8910	6	190.4	Shevchuk and Klimenko (2009)
	Bacillus subtilis	3.5	52.4	Nakajima and Tsuruta (2004)
	Citrobacter freudii	NA	48.02	Xie et al. (2008)
	Corynebacterium equi	3.5	21.4	Nakajima and Tsuruta (2004)
	Corynebacterium glutamicum	3.5	5.9	Nakajima and Tsuruta (2004)
	Micrococcus luteus	3.5	38.8	Nakajima and Tsuruta (2004)
	Zoogloea ramigera	3.5	49.7	Nakajima and Tsuruta (2004)

(continued)

Table 12.1 (continued)

Metal	Bacteria species	pH	Biosorption capacity ($mg\,g^{-1}$)	References
Th	*A. nicotianae*	3.5	75.9	Nakajima and Tsuruta (2004)
	B. licheniformis	3.5	66.1	Nakajima and Tsuruta (2004)
	B. megaterium	3.5	74.0	Nakajima and Tsuruta (2004)
	B. subtilis	3.5	71.9	Nakajima and Tsuruta (2004)
	C. equi	3.5	46.9	Nakajima and Tsuruta (2004)
	C. glutamicum	3.5	36.2	Nakajima and Tsuruta (2004)
	Micrococcus luteus	3.5	77	Nakajima and Tsuruta (2004)
	Zoogloea ramigera	3.5	67.8	Nakajima and Tsuruta (2004)
Pb	*Aeromonas hydrophila*	5	163.3	Hasan et al. (2009)
	B. cereus	NA	36.71	Jian-hua et al. (2007)
	C. glutamicum	5	567.7	Choi and Yun (2004)
	Enterobacter sp. J1	5	50.9	Lu et al. (2006)
	P. aeruginosa PU21	5	0.7	Lin and Lai (2006)
	P. aeruginosa ASU6a	7	79	Gabr et al. (2008)
	P. putida	5.5	270.4	Uslu and Tanyol (2006)
	R. opacus	5	0.455	Bueno et al. (2008)
	S. saprophyticus BMSZ71	5	184.89	Zamil et al. (2009)
	S. rimosus	NA	135	Selatnia et al. (2004c)

cell wall an overall negative charge, due to the presence of phosphodiester bonds between teichoic acid monomers. The highly charged nature of lipopolysaccharides confers an overall negative charge on the Gram-negative cell wall. The anionic functional groups present in the peptidoglycan, teichoic acids, and teichuronic acids of Gram-positive bacteria, and the peptidoglycan, phospholipids, and lipopolysaccharides of Gram-negative bacteria are the components primarily responsible for the anionic character and metal-binding capability of the cell wall (Moat et al. 2002; Prescott et al. 2002). Extracellular polysaccharides (EPSs) are also capable of binding metals; however, their availability depends on the bacterial species and growth conditions, and they can easily be removed by simple mechanical disruption or chemical washing (Yee and Fein 2001).

The cell walls of bacteria contain a large number of surface functional groups, in which carboxyl is generally the most acidic group in the bacteria. At low pH values, cell wall ligands are protonated and compete significantly with metals for binding. With increasing pH, more ligands, such as amino and carboxyl groups, could be exposed, leading to attraction between these negative charges and the metals, and hence increase biosorption onto the cell surface. Some bacteria have special structures, such as flagella and the S-layer. The S-layer is a surface and paracrystalline envelope present in several groups of bacteria and archaea. This layer is formed by protein or glycoprotein monomers that can self-assemble in two-dimensional structures (Sleytr et al. 2003). S-layers are associated with lipopolysaccharides

(LPSs) of Gram-negative bacteria or peptidoglycan of a Gram-positive cell (Urrutia 1997; Madigan et al. 2000). Porosity is between 30 and 70% and the diameter of the pore between 2 and 8 nm. This characteristic can be exploited for metal binding. An important characteristic of this protein is its capacity to reassemble once isolated from the cell (Pollmann et al. 2006). Due to this effect, it can be used for bioremediation. S-layer proteins might execute a trapping role of metallic ions in both living and dead cells, being a potential alternative for bioremediation of heavy metals in the field.

Some bacterial cells can produce a capsule outside the bacterial cell wall. They are highly hydrated and loosely arranged polymers of carbohydrates and proteins. Capsules are composed of polysaccharides and a few consist of proteins or polymers of amino acids called polypeptides (often formed from the D- rather than the L-isomer of an amino acid). *Bacillus anthracis*, the anthrax bacillus, can produce polypeptide capsules composed of D-glutamic acid subunits. Capsules may be thick or thin, rigid or flexible, depending on specific organism. Several different terms can be found to describe the capsule layer, such as slime layer, glycocalyx, and EPS. Capsule polymers are usually acidic in nature although capsules can consist of neutral polysaccharide, charged polysaccharide, or charged polypeptide. Capsule arrangement is important to metal binding (Madigan et al. 2000; Moat et al. 2002). The composition of bacterial EPS is complex, depending on the strain and its culture conditions. EPS synthesis is also reported for several pseudomonads, *Zoogloea ramigera*, *Rhizobium* sp., *Klebsiella* sp., and *Bacillus* sp. Typical constituents of EPS are mainly polysaccharides and proteins, often accompanied by nucleic acids, lipids, or humic substances (Flemming and Wingender 2001; van Hullebusch et al. 2003). Generally, EPSs have a high molecular weight with an abundance of negatively charged functional groups (ligands), e.g., carboxyl, hydroxyl, and uronic acids (Sobeck and Higgins 2002; Yan et al. 2008). These ligands make it possible for EPS to capture metal ions through electrostatic interactions, forming multiple complexes (Pulsawat et al. 2003). Hence, EPSs have been recommended as a metal absorbent because of their extensive complexing capacity for heavy metals (Gutnick and Bach 2000). Recent studies from Yan et al. (2008) showed that the polymer from *Bacillus* sp. 19 possessed an affinity for copper.

12.3 Mechanisms of Biosorption

Localizing the metal deposition site within the biosorption biomass and understanding the metal sequestering mechanism, in combination with elucidation of the relevant metal solution chemistry and chemical structure of the metal deposition site, are all crucial aspects of the quest for an efficient biosorption process which should feature high metal selectivity and uptake. The attractive feature of biosorption is a certain specificity of the biosorbent for divalent and multivalent heavy

metal cations. Metal uptake may vary widely for different genera and even for different mutant strains within a species. The nutrient status of the organism, its physiological state, the age of cells, and availability of micronutrients during growth, as well as environmental conditions during the biosorption process (e.g., pH, temperature, and presence of other metal ions), are all important parameters affecting the performance of a biosorbent. Solution chemistry of the metal also plays an important role in biosorption.

Biosorption is caused by a number of different physicochemical mechanisms, depending on a number of external environmental factors as well as on the metal, its ionic form in solution, and on the type of a particular active binding site responsible for sequestering the metal. Biosorption consists of several mechanisms, mainly ion exchange, chelating, adsorption, and diffusion through cell walls and membranes, which differ depending on the species used, the origin and processing of the biomass, and solution chemistry.

Research is in progress to establish biosorption as a commercially viable technique to trap and accumulate metals. Biosorption can serve as a tool for the recovery of precious metals (e.g., from processing solutions or seawater) and for the elimination of toxic metals (particularly from industrial wastewaters). The driving force of ion exchange is primarily the attraction of the biosorbent for the sorbate (metal). Metals can be bound electrostatically or by complexation. Interactions between the solute (metal) and the solvent play a role insofar as less hydrophilic molecules have a lower affinity for the liquid phase and are therefore sorbed more easily. Adsorption and microprecipitation involve binding of electrically neutral metals without the release of a stoichiometric amount of previously bound ions. In microprecipitation, the driving force is interaction between the solute and the solvent, whereas in adsorption affinity between sorbent and sorbate is the driving force. Microprecipitation does not necessarily involve a bond between biomass and metal.

In the case of physicochemical interaction based on physical adsorption, ion exchange, and complexation between metal and functional groups of the cell surface, metal uptake does not depend on cellular metabolism. The mechanism by which a metal binds onto the cell surface most likely includes electrostatic interactions, van der Waals forces, covalent bonding, or some combination of these processes. Negatively charged groups such as carboxyl, hydroxyl, and phosphoryl groups of the bacterial cell wall adsorb metal cations by electrostatic forces. Tunali et al. (2006) indicate that the biosorption of lead and copper by *Bacillus* sp. (ATS-1) involve an ion-exchange mechanism. Since the main mechanism involved in biosorption is ion exchange, protons compete with metal cations for the binding sites and for this reason pH is the operational condition which influences the process most strongly (Schiewer and Volesky 2000). pH determines protonation/deprotonation of metal ion binding sites and thus influences the availability of site to the sorbate. By lowering pH, it is also possible to release metal ions from the binding site. This property is used for the recovery of metal cations and regeneration of biosorbent.

12.4 Techniques Used in Metal Biosorption Studies

Carboxyl groups are negatively charged and abundantly available, actively participate in binding of metal cations. Mishra and Doble (2008) indicated that carboxyl and amino groups were responsible for the binding of chromate. Kang et al. (2007) observed that amine groups protonated at pH 3 and attracted negatively charged chromate ions via electrostatic interaction. Potentiometric titrations can provide information on type and number of binding sites. Kang et al. (2007) titrated *Pseudomonas aeruginosa* and determined the p*K*a values of available binding sites. Jian-hua et al. (2007) successfully correlated the quantity of acidic groups present on *Bacillus cereus* biomass, determined via potentiometric titrations, with the metal uptake capacity.

The nature of the binding sites and their involvement during biosorption can be approximately evaluated using FTIR. Loukidou et al. (2004) analyzed the FTIR spectra of Cd^{2+} loaded and unloaded *Aeromonas caviae*. Several band transformations allowed the authors to predict the possible involvement of amino, carbonyl, carboxyl, and phosphate groups in the biosorption of Cd^{2+}. Cayllahua et al. (2009) used FTIR spectra to confirm the presence of amide, carboxyl, and phosphate groups in *Rhodococcus* sp. biomass. Energy dispersive X-ray (EDX) can provide information regarding the chemical and elemental characteristics of biomass. Tunali et al. (2006) analyzed both Pb^{2+} and Cu^{2+} loaded *Bacillus* sp. using EDX, and confirmed the involvement of an ion-exchange mechanism during biosorption. In order to elucidate the chemical nature of bacterial cell-bound lanthanum, Kazy et al. (2006) employed X-ray diffraction (XRD) analysis and confirmed the involvement of cellular carboxyl and phosphate groups in the binding of lanthanum by *Pseudomonas* biomass. SEM micrographs have aided researchers in analyzing cell surface morphology before and after biosorption. Tunali et al. (2006) visualized the surface of metal-loaded *Bacillus* sp.

12.5 Factors Affecting Heavy Metal Biosorption

12.5.1 pH

Since the main mechanism involved in biosorption is ion exchange, protons compete with metal cations for the binding sites and for this reason pH is the operational condition which influences the process most strongly (Schiewer and Volesky 2000). The different chemical species of a metal occurring at different pH values will have variable charges and adsorbability at solid–liquid interfaces. In many instances, biosorption experiments conducted at alkaline pH values have been reported to complicate the evaluation of biosorbent potential as a result of metal precipitation (Selatnia et al. 2004c; Iqbal et al. 2007). pH

determines the speciation and solubility of toxic metal ions and also affects the properties of the biomass (Chen et al. 2008). Many metals occur as free hydrated species at lower pH, hydroxides are formed with increasing pH and eventually precipitation may occur. pH influences the magnitude of negative charge on the surface of the material by either protonation or deprotonation of metal-binding sites. The different pH sorption profiles for various heavy metal ions may be related to the nature of chemical interactions of each metal with biomass (Kiran et al. 2005; Bueno et al. 2008). For different biosorption systems of metal ions, the optimal pH will differ. Both cations and anions show different patterns of sorption on sorbent in the same pH range. Ma and Tobin (2004) reviewed that uptake of anions is favored at low pH with typical maximum biosorption in the range of 1–2 while cation biosorption is maximal at a higher pH range. Solution pH primarily affects the surface properties of the biomass (Antizar-Ladislao and Galil 2004). It is worth noting that the capability of microorganism biomass to adsorb or chelate metal ions is due to the presence of several chemical groups on the biomass surface which are polar or anionic in nature such as carboxyl, phosphate, amine, amino, hydroxyl, and sulfhydryl. Such groups will contribute to the electrokinetic potential (zeta potential) of the surface (Zouboulis et al. 1999). Different isoelectric points (i.e., pH value when net surface charge is zero) are exhibited by different microorganisms due to the differing chemical compositions of the cell wall. At pH lower than the isoelectric point, the overall charge of the biomass surface will become positive, whereas at pH higher than the isoelectric point, the overall surface charge will become negative (Zouboulis et al. 2004). In general, increasing pH increases the negative charge on the cell surface until all relevant functional groups are deprotonated, which favors electrochemical attraction and adsorption of cations. Furthermore, the increase in metal uptake with an increase in pH may be the result of more efficient competition of cations with H^+ for binding sites on bacteria (Ziagova et al. 2007; Green-Ruiz et al. 2008; Zamil et al. 2009). Anions would be expected to interact more strongly with cells with an increasing concentration of positive charges, due to the protonation of functional groups at lower pH values. Many papers discuss the effect of this factor on biosorption performance (Uslu and Tanyol 2006; Bueno et al. 2008; Gabr et al. 2008) by, e.g., determination of zeta potential, electrostatic attraction, and contribution of ion-exchange mechanisms (Xu et al. 2006).

Metal ions in solution undergo hydrolysis as the pH increases. The extent of hydrolysis at different pH values differs with each metal, but the usual sequence of hydrolysis involves the formation of hydroxylated monomeric species followed by the formation of polymeric species, and subsequently the formation of crystalline oxide precipitates after aging (Ziagova et al. 2007; Hasan and Srivastava 2009). The different chemical species of a metal that occur with pH changes vary in charge and adsorbability at solid–liquid interfaces. Therefore, adsorption of metals on interfaces is highly pH-dependent, and there is a critical pH range, usually of less than one pH unit, for each metal wherein the amount of metal adsorbed increases significantly.

12.5.2 Temperature

Biosorption by nonliving biomass is not significantly affected by the temperature. In contrast, the metabolism of living cells is temperature dependent, and hence change in this parameter will strongly affect the biosorption processes. Adsorption and ion exchange are exothermic in nature and hence the rate of these processes will increase with an increase in the temperature. However, at high temperatures, cell walls may be permanently damaged and for this reason a reduction in metal uptake is observed. Most of the increase in uptake has been reported in the temperature range of 4–23°C, whereas only a marginal increase is observed between 23 and 40°C. Metal uptake is reduced significantly when temperature is increased beyond this value. It is always desirable to conduct/evaluate biosorption at room temperature, as this condition is easy to replicate.

12.5.3 Initial Metal Ion Concentration

Initial solute concentration appears to have an impact on biosorption, with a higher concentration resulting in a high solute uptake (Öztürk 2007; Bueno et al. 2008; Uzel and Ozdemir 2009). This occurs because at lower initial solute concentrations, the ratio of the initial moles of solute to the available surface area is low; subsequently, the fractional sorption becomes independent of the initial concentration. However, at higher concentrations, the sites available for sorption become fewer compared with the moles of solute present and, hence, the removal of solute is strongly dependent upon initial solute concentration.

12.5.4 Initial Concentration of Biosorbent

The dosage of a biosorbent strongly influences the extent of biosorption. An increase in biomass concentration generally increases the amount of solute biosorbed, due to the increased surface area of the biosorbent, which in turn increases the number of binding sites (Ziagova et al. 2007; Bueno et al. 2008). Conversely, the quantity of biosorbed solute per unit weight of biosorbent decreases with an increasing biosorbent dosage, which may be due to the complex interaction of several factors. An important factor at high sorbent dosages is that the available solute is insufficient to completely cover the available exchangeable sites on the biosorbent, usually resulting in low solute uptake. The interference between binding sites due to increased biosorbent dosages cannot be overruled, as this will result in low specific uptake.

12.5.5 Presence of Competing Ions

Wastewaters usually contain multiple metals. The presence of more than one metal in wastewater is expected to cause interactive effects as a function of many factors, such as the number of metals competing for binding sites, metal concentration, and biosorbent dose. Many biosorption studies have been conducted with single-metal ion species in aqueous solutions. Metal uptake is significantly affected by the presence of other co-ions, as they will also compete for binding sites because many of the functional groups present on the cell wall and membrane are nonspecific. Therefore, metal uptake from mixed solutions is often found to be lower than those in a single-species system. Generally, metal uptake increases as the ionic radius of metal cation increases, with metals having higher ionic charge showing greater binding to biomass. Furthermore, the extent of reduction in metal uptake in the presence of other cations is found to be dependent on concentrations of the other cations. In particular, as the concentration of other cations increases, uptake of the metal further decreases. Bueno et al. (2008) reported that the presence of co-ions, whether in binary or ternary combinations, decreased the metal uptake when compared with the single-metal system. They observed that the presence of copper ions resulted in inhibition of lead uptake, which was greater than inhibition measured in the presence of chromium and copper ions together. In the presence of other metal ions in solution, chemical interactions between these species as well as with biomass may take place, resulting in competition for adsorption sites on the surface. As a consequence, the first component has a smaller "parking space" and its uptake is decreased (Akar et al. 2005).

Among the factors that affect biosorption preferences of a sorbent, binding of metal ions on biomaterials largely depends on physicochemical properties of the metallic species. It has been reported that the metal removal increases with the increase in ionic radius (Sag et al. 2002), which follows the order Pb(II) > Cu(II) > Cr(III). The differences in sorption affinities may also be attributed to differences in the electronegativity of the atoms, which also follows the order Pb(II) > Cu(II) > Cr(III). The greater the electronegativity or ionic radius, the greater the affinity, which also explains the significant suppression of lead uptake in the presence of copper and the moderate effect of chromium on lead biosorption. Uslu and Tanyol (2006) observed that the competitive biosorption capacities of *Pseudomonas putida* for Pb and Cu ions were lower than that under noncompetitive conditions.

Low atomic weight metal ions, such as Ca^{2+}, Na^{+}, and K^{+}, occur in industrial wastewater. The experimental data has shown that these metal ions have little effect on heavy metal biosorption, indicating low biomass affinity for the lighter ions. The presence of anions also affects biosorption of metal ions. Kapoor and Viraraghavan (1997) reported that biosorption capacity decreased in the presence of ethylenediamine tetraacetate (EDTA), sulfate, chloride, phosphate, carbonate, glutamate, citrate, and pyrophosphate. The anions in solution may form a complex

with the metal ions, which would significantly reduce metal biosorption capacity. In general, biosorption is reduced at increased ligand concentrations.

12.6 Development of Bacterial Biosorbents

Feasible approaches leading to improvement of heavy-metal biosorption efficiency include the development of more powerful biosorbents and the design of more efficient biosorption processes. Biosorbent development could be achieved by either isolating organisms with high capacity or high specificity to heavy metals or by tailoring genetically modified organisms abundant in high-affinity metal-binding proteins or polypeptides (Bae et al. 2002; Pazirandeh et al. 1998; Huang et al. 2003). Bae et al. (2003) reported that the metalloregulatory protein, MerR, which exhibits high affinity and selectivity toward mercury, was exploited for the construction of microbial biosorbents specific for mercury removal. Expression of mer operon genes encoding for cysteine-containing mercuric ion transport proteins (such as periplasmic protein MerP or inner membrane protein MerT) (Huang et al. 2003, Zhao et al. 2005) on *E. coli* is very effective biosorbents for heavy metal removal. In addition, several other metal-binding proteins, such as metallothioneins (MTs) (Kao et al. 2006), phytochelatins (PCs) (Grill 1987), and metal-binding peptides (Huang et al. 2003) were also expressed on *E. coli* to create powerful biosorbents. The MerP protein is a target for the development of genetically engineered biosorbents (Chen et al. 1998). Kao et al. (2008) used recombinant *E. coli* biosorbents with overexpression of MerP proteins for the biosorption of copper, nickel, and zinc from aqueous solutions. Deng et al. (2008) demonstrated biosorption by immobilized recombinant cells expressing human metallothionein proteins. Samuelson et al. (2000) generated recombinant *Staphylococcus xylosus* and *Staphylococcus carnosus* strains with surface-exposed chimeric proteins containing polyhistidyl peptides. Both strains of staphylococci gained improved nickel-binding capacities due to the introduction of H1 or H2 peptide into their surface proteins.

As the biosorption process is involved in mainly cell surface sequestration, modification of the cell wall can greatly alter the binding of metal ions. A number of methods have been employed for cell wall modification of microbial cells in order to enhance the metal-binding capacity of biomass and to elucidate the mechanism of biosorption. Physical treatments include heating/boiling, freezing/thawing, drying, and lyophilization. The various chemical treatments used for biomass modification include washing biomass with detergents, cross-linking with organic solvents, and alkali or acid treatment. Pretreatments could modify the surface characteristics/groups either by removing or masking the groups or by exposing more metal-binding sites (Vijayaraghavan and Yun 2008a, b). For example, grafting of long polymer chains onto the biomass surface through direct grafting or polymerization of a monomer could introduce functional groups onto the surface of biomass.

12.7 Biosorption and Equilibrium Studies of Heavy Metals

The type of process governs the rate of biosorption, which is considered as a rapid physical/chemical process. Biosorption can also be defined as a collective term for a number of passive accumulation processes, which may include ion exchange, coordination, complexation, chelation, adsorption, and microprecipitation. In equilibrium, a certain relationship prevails between solute concentration in solution and adsorbed state (i.e., the amount of solute adsorbed per unit mass of adsorbent). The equilibrium concentrations are a function of temperature; therefore, the adsorption equilibrium relationship at a given temperature is referred to as an adsorption isotherm. Several adsorption isotherms originally used for gas-phase adsorption are available and have been adopted to correlate adsorption equilibria in heavy metals biosorption. Some of the common equilibria are Freundlich, Langmuir, Redlich–Paterson, and the Sips equation. Freundlich and Langmuir equations are the most widely used. These isotherms for the removal of heavy metals from water and wastewater by biosorbents are discussed below.

12.7.1 Freundlich Isotherm

The Freundlich isotherm is an empirical equation and the most widely used isotherm for the description of adsorption equilibrium. It describes the adsorption of organic and inorganic compounds on a wide variety of adsorbents including biosorbents. The equation is written as:

$$q_e = K_F C_e^{\frac{1}{n}}, \tag{12.1}$$

where q_e is the amount adsorbed, K_F the characteristic constant related to the adsorption capacity, C_e the equilibrium concentration, and n the characteristic constant related to adsorption intensity or degree of favorability of adsorption.

Equation (12.1) can also be expressed in the linearized logarithmic form:

$$\log q_e = \log K_F + \frac{1}{n} \log C_e. \tag{12.2}$$

The plot of $\log q_e$ versus $\log C_e$ has a slope with the value of $1/n$ and an intercept magnitude of $\log K_F$. $\text{Log } K_F$ is equivalent to $\log q_e$ when C_e equals unity. However, in other cases when $1/n = 1$, the value of K_F depends on the units upon which q_e and C_e are expressed. A Freundlich constant n between 1 and 10 indicates favorable adsorption. A larger value of n (smaller value of $1/n$) implies stronger interaction between biosorbent and heavy metal while $1/n$ equal to 1 indicates linear adsorption leading to identical adsorption energies for all sites (Site 2001). The Freundlich isotherm has the ability to fit nearly all experimental adsorption–desorption data, and is excellent for fitting data from highly heterogeneous sorbent systems.

A $1/n$ value higher than unity ($n < 1$) suggests the presence of a curved upward isotherm, sometimes termed as a solvent-affinity type isotherm (Site 2001). Within this type of isotherm, the marginal sorption energy increases with increasing surface concentration. Sorption of solute on any sorbent can occur either by physical bonding, ion exchange, complexation, chelation or through a combination of these interactions. In the first case of physical bonding, as the solute is loosely bound, it can easily be desorbed using distilled water. Given the fact that miscellaneous functional groups such as hydroxyl, carbonyl, carboxyl, sulfhydryl, thioether, sulfonate, amine, imine, amide, imidazole, phosphonate, and phosphodiester groups can be present within the structure of the biosorbent, the mechanism of adsorption will not be restricted to physical bonding (Dursun 2006; Hanif et al. 2007; Wang et al. 2006a; Agarwal et al. 2006; Al-Rub 2006; Amarasinghe and Williams 2007; Basha et al. 2008; Liu et al. 2007; Parvathi et al. 2007; Popuri et al. 2007; Baral et al. 2007; Gokhale et al. 2008; Vijaya et al. 2008; Calfa and Torem 2008; Schiewer and Patil 2008; Kumar et al. 2008). Different mechanisms can be involved as the interaction between sorbent and solute molecules is expected to be strong.

Adsorption capacity is the most important characteristic of an adsorbent. It is defined as the amount of adsorbate taken up by the adsorbent per unit mass of adsorbent. This variable is governed by a series of properties, such as pore and particle size distribution, specific surface area, cation exchange capacity, pH, surface functional groups, and temperature.

As a precautionary note, the Freundlich equation is unable to predict the adsorption equilibria data at extreme concentrations. Furthermore, this equation is not reduced to linear adsorption expression at very low concentrations. However, researchers rarely face this problem, as moderate concentrations are frequently used in most biosorption studies.

12.7.2 Langmuir Isotherm

Another popular model for describing heavy metal sorption to biosorbents is the Langmuir model. The Langmuir equation relates the coverage of molecules on a solid surface to concentration of a medium above the solid surface at a fixed temperature. This isotherm is based on three assumptions, namely, adsorption is limited to monolayer coverage, all surface sites are alike and can only accommodate one adsorbed atom, and the ability of a molecule to be adsorbed on a given site is independent of its neighboring site's occupancy. By applying these assumptions, and a kinetic principle (rate of adsorption and desorption from the surface is equal), the Langmuir equation can be written in the following form:

$$q_e = \frac{q_{max}\,(K_L C_e)}{1 + K_L C_e}, \tag{12.3}$$

where q_e is the amount adsorbed, C_e the equilibrium concentration, q_{max} the saturated monolayer adsorption capacity, and K_L the sorption equilibrium constant.

This equation is often written in different linear forms (Ho 2006, Ho and Ofomaja 2006):

$$\frac{C_e}{q_e} = \frac{1}{q_{max} C_e} + \frac{1}{K_L q_{max}}, \tag{12.4}$$

$$\frac{C_e}{q_e} = \left(\frac{1}{K_L q_{max}}\right)\frac{1}{C_e} + \frac{1}{q_{max}}, \tag{12.5}$$

$$q_e = q_{max} - \left(\frac{1}{K_L}\right)\frac{q_e}{C_e}, \tag{12.6}$$

$$\frac{q_e}{C_e} = K_L q_{max} - K_L q_e. \tag{12.7}$$

In biosorption process, the saturation limit of certain biomass is affected by several factors such as the number of sites in the biosorbent material, accessibility of the sites, chemical state of the sites (i.e., availability), and affinity between site and metal (i.e., binding strength). In the case of covalent metal binding, supposing that an occupied site is theoretically available, the extent to which the site is to be occupied by a given metal depends further on its binding strength and concentration when compared with the metals already occupying the site.

The decrease of K_L value with an increase in temperature signifies the exothermicity of the adsorption process (physical adsorption) (Ho 2006; Padmavathy 2008; Djeribi and Hamdaoui 2008; Shaker 2007), while the opposite trend indicates that the process needs thermal energy (endothermic), leading to chemisorption (Dursun 2006; Ho 2006; Malkoc and Nuhoglu 2005; Wang et al. 2006b; Deng et al. 2006; Dundar et al. 2008; Aydin et al. 2008; Gupta and Rastogi 2008; Green-Ruiz et al. 2008; Vilar et al. 2008). During physical adsorption, the bonding between heavy metals and active sites of the biosorbent weakens at higher temperatures in contrast to chemisorption bonding, which becomes stronger. The exothermicity or endothermicity of the biosorption process can be determined via the heat of adsorption. This thermodynamic property is commonly obtained through an integrated Van't Hoff equation, which relates the Langmuir constant, K_L, to the temperature:

$$K_L = K_o \exp\left(-\frac{E_a}{RT}\right), \tag{12.8}$$

where K_o is the adsorption equilibrium constant, E_a the activation energy of adsorption/heat of adsorption, R the gas constant (0.0083 kJ/(mol K)), and T the absolute temperature (K).

12.7.3 Temkin Isotherm

The derivation of Temkin isotherm is based on the assumption that the decline of heat of sorption as a function of temperature is linear rather than logarithmic, as implied in the Freundlich equation (Basha et al. 2008; Isik 2008). The Temkin isotherm has the form:

$$q_e = \frac{RT}{b} \ln(aC_e), \tag{12.9}$$

where b is the Temkin constant in relation to heat of sorption (kJ/mol) and a the Temkin isotherm constant (L/g).

Several experimental studies in chemisorption systems are correlated using this equation (Mondal et al. 2008; Isik 2008; Kiran and Kaushik 2008). Mondal et al. (2008) studied the biosorption of As, Fe, Mn, Cu, and Zn on *Ralstonia eutropha*. For several systems such as biosorption of Ni(II) by ureolytic mixed culture (Isik 2008) and biosorption of Cr(VI) by *Lyngbya putealis* exopolysaccharides (Kiran and Kaushik 2008), Temkin isotherms are incapable of predicting biosorption equilibria. Since the basis of derivation for the Temkin equation involves simple assumptions, the complex phenomenon involved in liquid-phase adsorption is not taken into account by this equation. As a result, this equation is often not suitable for the representation of experimental data in complex systems.

12.7.4 Dubinin–Radushkevich Equation

The Dubinin–Radushkevich (DR) equation is excellent for interpreting sorption equilibria for organic compounds (in gas-phase condition) in porous solids. The DR equation is rarely applied onto liquid-phase adsorption due to the complexities associated with other factors such as pH and ionic equilibria inherent in these systems. In addition, the solute–solvent interactions often render the bulk solution nonideal. The mathematical expression for the DR equation in the liquid-phase system is given below:

$$q_e = q_{max} \exp\left(-\left(\frac{RT \ln\left(\frac{C_e}{C_s}\right)}{\beta E_0} \right)^2 \right), \tag{12.10}$$

where β is a constant (proportional to the liquid molar volume) and E_0 the solid characteristic energy toward a reference compound.

By taking into account the energetically nonuniform surface, this equation is capable of describing biosorption data as well (Igwe and Abia 2007; Cabuk et al. 2007; Vijayaraghavan et al. 2006; Apiratikul and Pavasant 2008; Kiran and Kaushik 2008). One of the best features of the DR equation lies in the fact that it

is temperature dependent. If the adsorption data at different temperatures are plotted as the logarithm of the amount adsorbed against the square of potential energy, all suitable data shall, in general, lie on the same curve, termed the characteristic curve. This curve can later be utilized as an initial "tool" to measure the applicability of the DR equation in expressing adsorption equilibria data.

12.7.5 Brunauer–Emmer–Teller (BET) Model

In the Langmuir model, it was assumed that adsorption only occurs on the unoccupied adsorption sites. In the BET model, this restriction is removed. Supposing that the initial adsorbed layer can act as a substrate for further adsorption, then the isotherm, instead of leveling off to some saturated value at high concentrations, is able to rise indefinitely. The same kinetics concept proposed by Langmuir is applied to this multiple layering process, i.e., the rate of adsorption on any layer is equal to the rate of desorption from that layer. The simplified form of BET equation can be written in the following form:

$$q_e = q_{max} \frac{BC_e}{(C_e - C_s^*)[1+(B-1)(C_e / C_s^*)}, \tag{12.11}$$

where B is a constant related to the energy of adsorption and C_s^* the saturation concentration of solute (mg/L).

Kiran and Kaushik (2008) showed a superb applicability of this model for Cr(VI) biosorption using *L. putealis* exopolysaccharides. They claimed that multilayer adsorption occurred in this system. As a note, other ideal assumptions within this model, namely, all sites are energetically identical along with no horizontal interaction between adsorbed molecules, may be correct for heterogeneous material and simple nonpolar gases but not for complex systems involving heterogeneous adsorbent such as biosorbents and metals. For that reason, this equation is unpopular in the interpretation of liquid-phase adsorption data for complex solids.

12.7.6 Redlich–Paterson Isotherm

Redlich–Paterson is another empirical equation, designated as the "three parameter equation," which is capable of representing adsorption equilibria over a wide concentration range. This equation has the following form:

$$q_e = \frac{K_{RP} C_e}{1 + a_{RP} C_e^{\beta}}, \tag{12.12}$$

where a_{RP}, K_{RP}, and β are Redlich–Paterson's parameters.

Equation (12.12) reduces to a linear isotherm at low surface coverage and to the Langmuir isotherm when β is equal to 1. This equation is quite popular for the prediction of heavy metal biosorption equilibria data (Dursun 2006; Basha et al. 2008; Ho and Ofomaja 2006; Ho 2006; Preetha and Viruthagiri 2007; Padmavathy 2008; Vijayaraghavan et al. 2006). Redlich and Paterson incorporated the characteristics of Langmuir and Freundlich isotherms into a single equation. Two limiting behaviors exist, i.e., the Langmuir form for $\beta=1$ and Henry's law form for $\beta=0$.

12.7.7 Multicomponent Heavy Metals Biosorption

The majority of the studies on biosorption of heavy metal ions by various biosorbents have focused on single-metal uptake. However, contrary to this, various metals are present in wastewater. The equilibrium modeling of multimetal biosorption, which is essential in the design of treatment systems, is often neglected. The effects of binary metal ions in various combinations seem to be more representative than single-metal studies (Aksu et al. 2002).

One of the major concerns arising from the adsorption of heavy metals from wastewater is the simultaneous presence of miscellaneous metals in wastewater. The interference and competition among different metals, metals and solvents, as well as metals and adsorption sites are significantly enough to be taken into account, leading to a more complex mathematical formulation of the equilibrium. Given the adsorption of heavy metals in real systems involving more than one component, adsorption equilibria involving competition between molecules of different types are warranted for better understanding of the system and design purposes. Only a few isotherms were developed to describe equilibrium in such systems. These models range from simple equations associated only with the individual isotherm parameters (nonmodified adsorption models) to more complex models exploiting the individual isotherm parameters along with correction factors (modified adsorption models) (Aksu et al. 2002).

Multicomponent adsorption models such as the multicomponent Langmuir model including its modification as well as the multicomponent Freundlich model have become popular. The multicomponent Langmuir model is expressed in the following form:

$$q_{e,i} = q_{\max,i} \frac{K_{L,i}\left(\frac{C_{e,i}}{\eta_i}\right)}{1+\sum_{j=1}^{N} K_{L,j}\left(\frac{C_{e,j}}{\eta_j}\right)}. \tag{12.13}$$

12.8 Kinetics of Heavy Metal Biosorption

Adsorption equilibria studies are important for determining the efficacy of metal adsorption. In addition, it is necessary to identify the adsorption mechanism type in a given system. For the purpose of investigating the mechanism of biosorption and

its potential rate-controlling steps that include mass transfer and chemical reaction processes, kinetic models have been used to test the experimental data. In addition, information on the kinetics of metal uptake is required to select the optimum conditions for full-scale batch metal removal processes.

Adsorption kinetics is expressed as the solute removal rate that controls the residence time of the sorbate in the solid–solution interface. Several adsorption kinetic models have been described for the adsorption kinetics and rate-limiting step. These include pseudo-first and -second-order rate models, the Weber and Morris sorption kinetic model, the Adam–Bohart–Thomas relation (Djeribi and Hamdaoui 2008), the first-order reversible reaction model (Baral et al. 2006), the external mass transfer model (Apiratikul and Pavasant 2008), the first-order equation of Bhattacharya and Venkobachar (Sag and Aktay 2002), and Elovich's model and Ritchie's equation. The pseudo-first and -second-order kinetic models are the most widely used models for biosorption kinetics of heavy metals and quantify the extent of uptake in biosorption kinetics.

12.8.1 Pseudo-First-Order Kinetics

The Lagergren first-order rate expression based on solid capacity is generally expressed as follows:

$$\frac{\mathrm{d}q}{\mathrm{d}t} = k_1(q_\mathrm{e} - q), \tag{12.14}$$

where q is the amount adsorbed at time t and k_1 the rate constant of first-order adsorption.

Integration of (12.14) with the boundary conditions as $t=0$, $q=0$, and at $t=t$, $q=q$, gives:

$$\ln\left(q_\mathrm{e} - q\right) = \ln q_\mathrm{e} - k_1 t. \tag{12.15}$$

Equation (12.15) can be written in the nonlinear form:

$$q = q_\mathrm{e}\left(1 - \exp\left(-k_1 t\right)\right). \tag{12.16}$$

Hypothetically, to ascertain the rate constants and equilibrium metal uptake, the straight-line plots of $\log(q_\mathrm{e} - q)$ against t of (12.15) were made at different initial metal concentrations (Ho and McKay 2002). The q_e value acquired by this method is then compared with the experimental value. If large discrepancies are posed, the reaction cannot be classified as first-order although this plot has a high correlation coefficient from the fitting process. Nonlinear fitting of (12.16) is another way to achieve the predicted value of q_e and k_1, although this is not a common exercise. The trend shows that the predicted q_e values seem to be lower than the experimental values. A time lag, probably caused by the presence of a

boundary layer or external resistance controlling the beginning of the sorption process, was argued to be the responsible factor behind the discrepancy (Vijayaraghavan et al. 2006).

12.8.2 Pseudo-Second-Order Kinetics

The pseudo-second-order model is derived on the basis of the sorption capacity of the solid phase, expressed as:

$$\frac{dq}{dt} = k_2(q_e - q)^2, \tag{12.17}$$

where k_2 is the rate constant for pseudo-second-order model. Integration of (12.17) with the boundary conditions $t=0$, $q=0$, and at $t=t$, $q=q$, results in:

$$\frac{1}{q_e - q} = \frac{1}{q_e} + k_2 t. \tag{12.18}$$

Equation (12.18) can be stated in the linear form as:

$$\frac{t}{q} = \frac{t}{q_e} + \frac{1}{k_2 q_e^2}. \tag{12.19}$$

The pseudo-second-order rate constants were determined experimentally by plotting t/q against t. Ho (2006) conducted an evaluation using linear and nonlinear methods to determine the pseudo-second-order kinetic parameters. He chose cadmium as the heavy metal and tree fern as the biosorbent. As-acquired kinetic parameters from four kinetic linear equations using the linear method have discrepancies among themselves. Further, for the linear method, the pseudo-second-order model as written in (12.19) has the highest coefficient of determination. In contrast to the linear model, the resulting kinetic parameters from the nonlinear model were almost identical among each other. To that end, the nonlinear method is considered as a better way to ascertain the desired parameters. Still, most of the biosorption studies in the literatures utilize (12.19).

As such, in comparison to pseudo-first-order kinetics, this model is considered more appropriate to represent the kinetic data in biosorption systems. This tendency comes as an indication that the rate-limiting step in biosorption of heavy metals are chemisorption involving valence forces through the sharing or exchange of electrons between sorbent and sorbate (Javed et al. 2007; Ofomaja and Ho 2007; Nasreen et al. 2008; Namasivayam and Sureshkumar 2008; Dundar et al. 2008; Yu et al. 2007; Rahaman et al. 2008; Mack et al. 2008; Pamukoglu and Kargi 2007; Miretzky et al. 2008; Guo et al. 2008), complexation, coordination, and/or chelation (Yu et al. 2007; Baral et al. 2007; Lu and Gibb 2008).

12.8.3 The Weber and Morris Sorption Kinetic Model

The Weber and Morris (WM) sorption kinetic model was initially employed by Pasavant et al. (2006) to describe their biosorption experimental data. This model has the following form:

$$q = K_{\mathrm{WM}}\sqrt{t}, \tag{12.20}$$

where K_{WM} is the Weber and Morris intraparticle diffusion rate.

In their investigation, the sorption process by biomass for Cu(II), Cd(II), Pb(II), and Zn(II) was regulated by two main mechanisms, i.e., intraparticle diffusion and external mass transfer. The intraparticle diffusion can be estimated with the following equation:

$$D = \frac{\pi}{8640}\left(\frac{(d_{\mathrm{p}}K_{\mathrm{WM}})}{q_{\mathrm{e}}}\right)^2, \tag{12.21}$$

where d_{p} is the mean particle diameter.

The external mass transfer process was determined by:

$$\frac{\mathrm{d}q}{\mathrm{d}t} = K'_{\mathrm{L}}A(C - C_{\mathrm{s}}^{i}), \tag{12.22}$$

where K'_{L} is the liquid–solid mass transfer coefficient, A the specific surface area of biomass, C the liquid-phase concentration of sorbate in the bulk solution at t, and C_{s}^{i} the concentration of sorbate in the inner pore of sorbent.

They observed that the external mass transfer coefficients can be ordered from high to low values as Cu(II) > Pb(II) > Zn(II) > Cd(II) while the intraparticle diffusion coefficients (also in the decline sequence) as Cd(II) > Zn(II) > Cu(II) > Pb(II).

12.8.4 First-Order Reversible Reaction Model

To derive this model, the sorption of metal on biosorbent is assumed to be a first-order reversible reaction, as expressed by the following reaction mechanism (Baral et al. 2006):

$$\mathrm{A} \Leftrightarrow \mathrm{B} \tag{12.23}$$

In turn, the rate equation for the reaction is expressed as:

$$\frac{\mathrm{d}C_{\mathrm{B}}}{\mathrm{d}t} = -\frac{\mathrm{d}C_{\mathrm{A}}}{\mathrm{d}t} = k_1^0 C_{\mathrm{A}} - k_2^0 C_{\mathrm{B}} = k_1^0\left(C_{\mathrm{A0}} - C_{\mathrm{A0}}X_{\mathrm{A}}\right) - k_2^0(C_{\mathrm{B0}} - C_{\mathrm{A0}}), \tag{12.24}$$

where C_{B} is the concentration of metal in sorbent at time t, C_{A} the concentration of metal in solution at time t, k_1^0 and k_2^0 the first-order rate constants, C_{A0} the initial concentration of adsorbate, C_{B0} the initial concentration of adsorbent, C_{Be} the equilibrium

concentration of metal in adsorbent, and C_{Ae} the equilibrium concentration of metal in adsorbate.

At equilibrium conditions:

$$K_c = \frac{C_{Be}}{C_{Ae}} = \frac{k_2^0}{k_1^0}. \tag{12.25}$$

Integrating (12.24) and applying the equilibrium condition gives:

$$\ln\left(\frac{-\left(C_{AO} + C_A\right)}{C_{AO} + C_{Ae}}\right) = -\left(k_1^0 + k_2^0\right)t. \tag{12.26}$$

Baral et al. (2006) tried several equations to represent the Cr^{6+} biosorption experimental data, and one among these equations was first-order reversible reaction model. This equation fits well for their experimental data. The reduced rate constants and increasing equilibrium constant with the rise in temperature signifies that the biosorption of Cr^{6+} onto treated sawdust has exothermic nature. These observations, however, suggesting a complication as a careful examination onto the rate constant parameters revealed an existing violation toward Le Chatelier's principle. Since the adsorption process is exothermic as a rule, the rate constant value of k_1^0 should decrease with increasing temperature. Based on the Le Chatelier's principle, if the adsorption is exothermic, desorption would be endothermic. Therefore, the rate constant value of k_2^0 should be enhanced in parallel with the rise in temperature. As mentioned previously, sorption of heavy metals on any biosorbent takes place by either physical bonding, ion exchange, complexation, coordination/chelation or a combination of these. By being restricted to a reversible chemical reaction assumption, this model fails to capture any other possible complex mechanism involved.

12.9 Immobilization of Bacteria

In addition to the high biosorption yield obtained by bacteria, the heavy metal bioremediation process requires microorganisms to be attached to a solid surface. Surface fixation and cell entrapment are the two methods of immobilization. Different matrices were tested for cell immobilization (Beolchini et al., 2003; Xiangliang et al., 2005). Support matrices suitable for biomass immobilization include alginate, polyacrylamide, polyvinyl alcohol, polysulfone, silica gel, cellulose, and glutaraldehyde (Wang 2002; Vijayaraghavan and Yun 2008a, b). The polymeric matrix determines the mechanical strength and chemical resistance of the final biosorbent particle to be utilized for successive sorption–desorption cycles, so it is important to choose the correct immobilization matrix. Akar et al. (2009) measured the biosorption of 100 mg L^{-1} of nickel at pH 6.5 to be 33.83 and 7.50 mg g^{-1} for silica gel and *Proteus vulgaris*, respectively, whereas the immobilized biosorbent had a biosorption capacity of 45.48 mg g^{-1} under the same conditions. Maximum biosorption obtained using immobilized biomass provides promise for immobilized cells in a column reactor for

the remediation of heavy metals. At pH 5.0, the Cd^{2+} biosorption capacity of *E. coli* biomass-free PVA beads was 1.30 mg g^{-1}, which was significantly lower than the adsorption capacity of PVA-immobilized cells, displaying a capacity of 2.18 and 4.41 mg/g for biomass loading of 8.42 and 19.5 wt%, respectively (Kao et al. 2009).

Although cell entrapment imparts mechanical strength and resistance to chemical and microbial degradation upon the biosorbent, the costs of immobilizing agent cannot be ignored. Free cells are not suitable for use in a column, due to their low density and size they tend to plug the bed, resulting in marked declines in pressure. For industrial applications of biosorption, it is important to utilize an appropriate immobilization technique to prepare commercial biosorbents which retain the ability of microbial biomass to adsorb metal(s) during the continuous treatment process. The immobilization of biomass in solid structures would create a biosorbent material with the right size, mechanical strength, rigidity, and porosity necessary for use in practical processes. The immobilized materials can be used in a manner similar to ion-exchange resins and activated carbon such as adsorption–desorption cycles (i.e., recovery of the adsorbed metal, reactivated and reuse of the biomass) (Veglio and Beolchini 1997).

In different matrices, tested surface fixation was chosen as the immobilization methodology instead of cell entrapment. Cell immobilization has successfully been achieved mostly in calcium alginate beads, but this matrix also has a high affinity for heavy metals. Metal retention kinetics studies with calcium alginate confirmed that almost 100% of the metal assayed was retained by the beads (Vullo et al. 2003) and that it is pointless to try to improve heavy metal retention by bacterial cell entrapment in calcium alginate beads (Arica et al. 2001; Davis et al. 2003; Vullo et al. 2003; Arica et al. 2004). Although calcium alginate is useful for entrapping cells in its gel structure, its advantage resides mostly in the re-utilization of the entrapped cells. However, the high heavy metal affinity of alginate makes it unusable for the development of continuous industrial processes, as the recovery of the alginic acid would increase the final costs of effluent treatment. Successful bacterial immobilization was achieved on inert surfaces such as Teflon membranes, silicone rubber, and polyurethane foams. Best results of surface fixation were obtained with *Pseudomonas veronii* 2E, which was able to grow on all three surfaces. This organism developed a film over the matrix surfaces, and also formed aggregates and adhered to glass during batch culture work. The development of other bacteria on the same surfaces was barely observed.

12.10 Desorption of Heavy Metals

Biosorption is a process of treating pollutant-bearing solutions to render it contaminant-free. However, it is also necessary to be able to regenerate the biosorbent. This is possible only with the aid of appropriate elutants which usually results in a concentrated pollutant solution. Therefore, the overall achievement of a biosorption process is to concentrate the solute, i.e., sorption followed by desorption. Desorption is of utmost importance when biomass preparation/generation is costly, as it is possible to decrease process cost and the dependency of the process on a continuous

supply of biosorbent. A successful desorption process requires the proper selection of elutants which strongly depends on the type of biosorbent and the mechanism of biosorption. In addition, the elutant must be (1) nondamaging to the biomass, (2) less costly, (3) environmental friendly, and (4) effective. Several investigators have conducted exhaustive screening experiments to identify appropriate elutants for this process. Kuyucak and Volesky (1989) examined several chemical agents to desorb Co^{2+} from cobalt-laden *Ascophyllum nodosum*, and identified $CaCl_2$ in the presence of HCl as a suitable elutant.

The performance of an elutant also strongly depends on the type of mechanism responsible for biosorption. For instance, electrostatic attraction was found to be the primary mechanism responsible for biosorption of negatively charged dye anions to a positively charged cell surface (O'Mahony et al. 2002). Therefore, it would be logical to make the cell surface negative using alkaline solutions to repel the negatively charged reactive dyes (Won and Yun 2008). Elution is also influenced by the volume of elutant, which should be as low as practically possible to obtain the maximum solute concentration in the smallest possible volume (Volesky 2001). At the same time, the volume of the solution should be sufficient to provide maximum solubility for the desorbed solute. Also, one has to realize that the desorbed sorbate stays in solution and a new equilibrium is established between that and the one (remaining) still fixed on the biosorbent. This leads to the concept of a "desorption isotherm" where the equilibrium is strongly shifted toward the sorbate dissolved in the solution (Yang and Volesky 1996). Thus, it is necessary to evaluate the suitable elutant volume, which can be performed using experiments with different solid-to-liquid ratios. The solid-to-liquid ratio is defined as the mass of solute-laden biosorbent to the volume of elutant. Davis et al. (2000) observed that solid-to-liquid ratio affected copper elution efficiency of $CaCl_2$ solutions, while it was nearly independent in the case of 0.1 M HCl. The purpose of desorption is to unbind a contaminant from a biosorbent, so both the recovered solute and biosorbent can be reused. After desorption, the biosorbent should be close to its original form, both morphologically and effectually. Also, during the desorption process, removal of all bound sorbate from biosorbent should be assured. If this does not occur, a diminished uptake should be expected in the next cycle. Puranik and Paknikar (1999) regenerated and reused a polysulfone-immobilized *Citrobacter* strain over three cycles for the biosorption of lead, cadmium, and zinc, using 0.1 M HCl and 0.1 M EDTA as elutants, but only with limited success, and emphasized the need for further screening work. Beolchini et al. (2003) immobilized *Sphaerotilus natans* into a polysulfone matrix for the biosorption of copper, and with the aid of 0.05 M $CaCl_2$ regenerated and reused the beads over ten cycles with satisfactory results.

12.11 Biosorption and Its Column Performance

Continuous biosorption studies are of utmost importance to evaluate the technical feasibility of a process for real applications. Among the different column configurations, packed bed columns have been established as an effective, economical, and

most convenient setup for biosorption processes (Zhao et al. 1999; Saeed and Iqbal 2003; Volesky et al. 2003; Chu 2004). These authors make best use of the concentration difference, which is known to be the driving force for sorption, and allow more efficient utilization of the sorbent capacity, resulting in better effluent quality (Aksu and Gönen 2004). Also, packed bed sorption has a number of process engineering merits, including a high operational yield and the relative ease of scaling-up procedures (Aksu 2005). Other column contactors, such as fluidized and continuous stirred tank reactors, are rarely used for biosorption (Prakasham et al. 1999; Solisio et al. 2000). Continuous stirred tank reactors are useful when the biosorbent is in the form of a powder (Cossich et al. 2004); however, they suffer from high capital and operating costs (Volesky 1987). Fluidized bed systems, which operate continuously, require high flow rates to keep the biosorbent particles in suspension (Muraleedharan et al. 1991). Various parameters can be used to characterize the performance of packed bed biosorption, including the length of the sorption zone, uptake, removal efficiency, and slope of the breakthrough curve (Volesky et al. 2003; Vijayaraghavan et al. 2004). A mass transfer zone will develop between the gradually saturated section of the column and the fresh biosorbent section (Naja and Volesky 2006). The length of this zone is important practically, which can be calculated from:

$$Z_{\mathrm{m}} = Z\left(1-\frac{t_{\mathrm{b}}}{t_{\mathrm{e}}}\right), \tag{12.27}$$

where Z denotes bed depth (cm), and t_{b} and t_{e} the column breakthrough and exhaustion times (h), respectively. The uptake is an important parameter often used to characterize the performance of a biosorbent in a packed column. The column uptake (Q_{col}) can be calculated by dividing the total mass of biosorbed sorbate (m_{ad}) by that of the biosorbent (M). The mass of biosorbed sorbate is calculated from the area above the breakthrough curve (C vs. t) multiplied by the flow rate. The removal efficiency (%) can be calculated, from the ratio of the sorbate mass biosorbed to the total mass of sorbate sent to the column, as follows:

$$\text{Removal efficiency } (\%) = \frac{m_{\mathrm{ad}}}{C_0 F t_{\mathrm{e}}} \times 100, \tag{12.28}$$

where C_0 and F are the inlet solute concentration (mg/L) and flow rate (L/h), respectively. It is important to note that the removal efficiency is independent of the biosorbent mass, but solely dependent on the flow volume. Therefore, it is necessary to consider both the uptake and removal efficiency when evaluating biosorbent potential.

The slope of the breakthrough curve from t_{b} to t_{e} ($\mathrm{d}c/\mathrm{d}t$) is often used to characterize the shape of the curve (Volesky et al. 2003). It is preferential to have an extended breakthrough curve with a steep slope, as it is usually the result of a shorter mass transfer zone, which implies a longer column service time and greater utilization of the sorbent portion inside the column (Kratochvil and Volesky 1998). Thus, for effective biosorbents, a delayed breakthrough, earlier exhaustion, shortened mass transfer zone, high uptake, steep breakthrough curve, and high removal efficiency would be expected.

12.11.1 Column Regeneration

Regeneration of biosorbent is relatively easier in a packed column arrangement, with the aid of an appropriate elutant. When the column becomes saturated, the contaminant solution flow should be switched to the elutant flow. In general, an elution process is usually rapid compared with that of sorption. Thus, a high contaminant concentration in a small elutant volume would be expected under optimized process conditions. In addition, it is desirable to limit the contact of the elutant with the sorbent. This is because extreme process conditions such as highly alkaline or acidic solutions are often employed for elution; thus, morphological damage to the biosorbent can be expected. Therefore, the optimal flow rate for the elution should be identified for successful reuse of the biosorbent over multiple cycles. In a typical elution curve, a sharp concentration increase is expected at the beginning, followed by a gradual decrease (Volesky et al. 2003; Vijayaraghavan et al. 2005).

Even with the successful optimization of an elution process, several investigators have observed a decrease in biosorption performance over subsequent cycles (Saeed and Iqbal 2003; Volesky et al. 2003; Vijayaraghavan et al. 2004). A loss of sorption performance during long-term use may occur for a variety of reasons, e.g., changes in the chemistry and structure of the biosorbent as well as flow and mass transport conditions within the column.

12.11.2 Sorption Column Model

The Bohart–Adams sorption model (Jansson-Charrier et al. 1996; Muraleedharan et al. 1994), developed primarily for carbon sorption, has often been used in studies of biosorption column performance; however, it is not an appropriate model that would reflect the uptake mechanism of ion exchange. The most complete column model taking into account dominant intraparticle mass transfer was developed for ion exchange by Tan and Spinner (1994). In principle, this mass transfer model can predict breakthrough curves for all species removed by the biosorbent and also the elution curves obtained during sorbent regeneration.

To predict biosorption in fixed-bed columns, the model based on the work of Kratochvil and Volesky (2000) is used. Adaptation of this model was necessary in order to study binary systems as well as ternary and quaternary systems. Its transformation allowed testing the modeling approach for the case of multicomponent biosorption systems. The adapted approach consisted of numerically solving a mixed system of partial differential, ordinary differential, and algebraic equations describing the dynamics of multicomponent ion exchange in a flow-through fixed bed. Assuming isothermal conditions and constant physical properties for the feed solution, the differential molar balance for a sorbate species M is:

$$\frac{C_{\mathrm{M}}}{\partial Z} - \frac{1}{\mathrm{Pe_c}} \frac{\partial^2 C_{\mathrm{M}}}{\partial Z} + \frac{\partial C_{\mathrm{M}}}{\partial \mathrm{t}} + D_{\mathrm{gM}} \frac{\mathrm{q_M}}{\mathrm{t}} = 0 \tag{12.29}$$

The sorption rate equation can be written as (12.30), assuming a linear driving force for the sorption process and a combined film and intraparticle mass transfer resistance:

$$\frac{\partial qM}{\partial t} = \mathrm{Sh_M}(q^*_{\mathrm{M}} - q_{\mathrm{M}}) \tag{12.30}$$

With

$$D_{\mathrm{gM}} = \frac{\rho bQ}{C_{0\varepsilon}}, \tag{12.31}$$

$$\mathrm{Sh_M} = \frac{K_{\mathrm{f}}\mathrm{M}^{L_0}}{v}, \tag{12.32}$$

$$P_{\mathrm{ec}} = \frac{L_0 v}{D_{\mathrm{z}}}, \tag{12.33}$$

where t is the time (h), ρb the packing density of dry biomass in the packed bed ($\mathrm{g\,L^{-1}}$), Q the concentration of binding sites in the biosorbent ($\mathrm{meq\,g^{-1}}$), C_0 the normality of the column feed ($\mathrm{meq\,L^{-1}}$), ε the column void fraction, L_0 the length of the column (cm), v the interstitial fluid velocity ($\mathrm{cm\,min^{-1}}$), D_Z the dispersion coefficient in the liquid phase ($\mathrm{cm^2\,min^{-1}}$), $\mathrm{Sh_M}$ the rate constant for ion exchange ($\mathrm{min^{-1}}$), C_{M} the concentration of species M in the liquid phase ($\mathrm{meq\,L^{-1}}$), q_{M} the uptake of species M by the biosorbent ($\mathrm{meq\,g^{-1}}$), q^*_{M} the dimensionless equilibrium uptake of species M at C_{M}, D_{gM} the solute distribution parameter, K_{fM} the overall mass transfer coefficient of species M ($\mathrm{min^{-1}}$), and P_{ec} is the column Peclet number.

12.12 Conclusion

Biosorption offers an economically feasible technology for efficient removal and recovery of metal(s) from aqueous solution. The process of biosorption has many attractive features including selective removal of metals over a broad range of pH and temperature, rapid kinetics of adsorption and desorption, and low capital and operational costs. Biosorbents can easily be produced using inexpensive growth media or obtained as a by-product from industry. The use of immobilized biomass rather than native biomass has been recommended for large-scale application, but various immobilization techniques have yet to be thoroughly investigated for ease, efficacy, and cost effectiveness. When designing a reactor for water treatment, it is important to achieve optimal conditions for metal retention at the lowest cost. Also, for an ex situ bioremediation process, costs will be lower when there is no need to include nutrients. The use of fixed or fluidized bed reactors is preferred because of easier recovery of the treated effluent, so successful bacterial immobilization on different matrices is required. Experiments in complexing capacity evaluation in

industrial effluents are necessary to verify metal bioavailability and improve the efficiency of the process. Although continuous processes of immobilized cells have been realized at laboratory scale, there is still a long way to go for biosorption commercialization. Selection of quality, inexpensive support materials for biomaterial immobilization, improvement of reuse methods, and enhancement of properties of immobilized biosorbents such as pore ratio, mechanical intensity, and chemical stability are also important factors for application (Hu and Wang 2003).

Biosorption processes are applicable to effluents containing low concentrations of heavy metals for an extended period. This aspect makes it even more attractive for the treatment of dilute effluent that originates either from an industrial plant or from a primary wastewater treatment facility. Thus, biomass-based technologies need not necessarily replace conventional treatment routes but may complement them. To provide an economically viable treatment, the appropriate choice of biomass and proper operational conditions must be identified.

Critical analysis reveals that not all metal-polluted wastewater-generating industries have the interest or the capability to treat effluents and most industries opt only for basic treatment techniques simply to comply with regulations. To attract greater usage of biosorption, strategies must be formulated to centralize facilities for accepting the used biosorbent where its processing can be carried out to either regenerate the biomass or convert the recovered metal into a usable form. This will further require an interdisciplinary approach with the integration of metallurgical skills along with sorption and wastewater treatment to apply biosorption technology for combating heavy metal pollution in aqueous systems.

12.13 Future Prospects

For the future of biosorption, there are two trends of development for the removal of metals. One is to use hybrid technology for pollutant removal, especially using living cells. A second trend requires the improvement of biomaterials immobilization, as well as optimization of the parameters of the biosorption process and physicochemical conditions, including reuse and recycling. Market factors for successful application of biosorption should be considered. The mechanisms involved in biosorption or metal–microbe interactions should be further studied. Molecular biotechnology, a powerful tool to elucidate mechanisms at the molecular level, should be considered more in the future to construct an engineered organism with higher sorption capacity and specificity for target metal ions.

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Chapter 13
Metal Tolerance and Biosorption Potential of Soil Fungi: Applications for a Green and Clean Water Treatment Technology

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Abstract Heavy metals pose a significant ecological and public health hazard because of their toxic effects and their ability to accumulate in terrestrial and aquatic food chains. This chapter addresses the interactions of heavy metals with organisms for application in wastewater or soil treatment systems, with special emphasis on yeasts and fungi. Conventional techniques to remove metals from wastewaters have several disadvantages; however, biosorption has demonstrated significant metal removal performance from large volumes of effluents. One key step of treatment processes for cleanup of heavy metal-enriched water or soil involves growing resistant cells that accumulate metals to optimize removal through a combination of biosorption and continuous metabolic uptake. Fungal biosorption can be used for the removal of metals from contaminated water and soil; fungal biosorbents are less expensive and more effective alternatives for the removal of metallic elements, especially heavy metals, from aqueous solution. In this chapter, the biosorption abilities of fungal biomass toward metal ions are emphasized. The chapter also highlights the mechanisms involved in fungal biosorption and the factors affecting the biosorption process. The current status and achievements of fungal biosorption technologies are reviewed.

13.1 Introduction

Rapid industrialization and urbanization have resulted in elevated emissions of toxic heavy metals and radionuclides to the biosphere. Inorganic toxicants may occur as cations of metals such as mercury (Hg), cadmium (Cd), chromium (Cr), lead (Pb), nickel (Ni), and uranium (U). Toxic inorganics may also include alkylated or aromatized forms of metal ions, such as methylmercury and phenylmercury.

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_13,

The increasing quantities of toxic metals emitted into the biosphere pose potential hazards to ecosystems and influence the metabolism of living organisms (Gazso et al. 2001; Ansari and Malik 2007).

Heavy metals pose a significant threat to the environment and public health because of their toxicity and their accumulation in soil and food chains (Ceribasi and Yetis 2001; Chen et al. 2009; Gurel et al. 2010). Metal pollution of the biosphere by toxic metals has accelerated dramatically since the industrial revolution (Mclveen and Negusanti 1994). Agricultural application of wastewater and sludge and improper disposal of industrial effluents in developing countries, including India, have resulted in the accumulation of toxic heavy metals in soil. Most heavy metals (except Cd, Hg, and Pb) are required by living organisms in trace quantities; however, at elevated concentrations these become environmental toxins. Once the soil is contaminated with a metal, it is difficult and costly to remove from soil. In addition, microbial diversity and their activity in soil will be adversely affected, which may result in adverse effects on soil productivity (Ansari and Malik 2010).

Industrialized countries are increasingly concerned regarding the occurrence of toxic metals in the environment. The most effective policy to minimize their release from industrial or agricultural sources is the adoption of low waste-generating technologies coupled with effective effluent purification processes (Fourest et al. 1994; Sag et al. 2000).

In recent years, public awareness has increased regarding the long-term effects of wastewater containing toxic elements. Numerous industrial processes generate aqueous effluents contaminated with heavy metals. Metal concentrations must be reduced to meet ever increasing legislative standards and recovered where feasible. According to the World Health Organization, the metals of most immediate concern are Hg, Cr, Cd, Pb, Ni, aluminum (Al), manganese (Mn), iron (Fe), cobalt (Co), copper (Cu), and zinc (Zn) (Allen and Brown 1995).

Removal of heavy metal ions from wastewater is necessary due to their toxic properties. Chemical precipitation, chemical oxidation and reduction, ion exchange, filtration, electrochemical treatment, reverse osmosis, evaporative recovery, and solvent extraction are the most commonly used procedures for removing heavy metal ions from aqueous environments (Ucun et al. 2003; Babu et al. 2007; Acheampong et al. 2010). However, these technologies have several disadvantages such as unpredictable rates of metal ion removal, high reagent or energy requirements, and/or generation of toxic sludge, which is often difficult to dewater and requires extreme caution in its disposal. The search for new and innovative treatment technologies has focused attention on the effects of heavy metal toxicity on, and uptake by, microorganisms (Aksu et al. 1997). Using microorganisms as biosorbents for heavy metals offers a potential alternative to existing methods (Igwe and Abia 2006; Malik 2004). With the growing scarcity and increasing economic value of certain metals, this intrinsic property of microorganisms has also given importance to the study of microbial metal recovery.

The use of biomass for heavy metal removal or recovery has gained importance in recent years due to its promising performance and low cost. Among the various sources, both live and inactivated biomass of microorganisms (fungi, algae, bacteria, etc.)

exhibit promising metal-binding capacities. Their complex cell walls contain high concentrations of functional groups including amino, amide, hydroxyl, carboxyl, sulfhydryl, and phosphate, which have been associated with metal binding (Akhtar and Mohan 1995; Gardea-Torresdey et al. 2004). Specific constituents of fungal cell walls, e.g. chitin, have been documented as possessing significant metal binding abilities (Gadd et al. 2001).

Fungi possess many properties that influence metal mobility and toxicity, including the production of metal-binding proteins, organic and inorganic precipitation, active transport, and intracellular compartmentalization.

The uptake of heavy metals by fungi is of industrial relevance (Gadd 1986a). Fungi are well suited for removal of metal ions from wastewater, since they exhibit marked tolerance toward metals and other adverse conditions, e.g., low pH. Fungi have higher capacities of metal binding to cell walls than other microorganisms.

The scientific literature indicates that the use of fungi and other microorganisms as biosorbents for heavy metals offers a potential alternative to existing chemical and physical methods, which possess several disadvantages. It is expected that filamentous fungi of heavy metal-contaminated habitats exhibiting significant tolerance to toxic metals and demonstrating metal-complexing metabolites or activity will serve as efficient biosorbents for heavy metals.

Considering the problem of heavy metal pollution and the importance of fungi as a potential biosorbent for heavy metals, the present chapter discusses the diversity of soil fungi, interactions with metals, and the development and exploitation of metal tolerance. The potential of various fungi in the biosorption of heavy metals from aqueous environments and their future prospects are discussed.

13.2 Soil Fungi and Their Diversity

Fungi are eukaryotic organisms and are ubiquitous members of soil microbial communities. They comprise a varying proportion of the overall biomass in different systems. Fungi tend to dominate in soils containing high proportions of organic matter and of low pH and generally constitute a smaller proportion in intensively managed mineral soils. The fungi are an immensely diverse group of organisms, encompassing a wide range of forms from microscopic single-celled yeasts to large macrofungi, as exemplified by the well-known mushrooms and toadstools and the largest of fruiting bodies, the giant puffball (Bridge and Spooner 2001).

The majority of fungal species occur in the soil environment at some stage in their life cycle. Current knowledge of fungal diversity in soil is based largely on observations of fruiting bodies or cultures, which are obtained from soil isolation studies. Both approaches have limitations for the detection of the true diversity in any chosen environment. Approximately 17% of the known fungal species can be successfully grown in culture. Detecting exactly which fungi are present in a soil sample is not an easy task, one of the major problems being the fastidious nature of the great majority of species (Hawksworth et al. 1995). If the above figure was applied to the

1,200 culturable fungi species occurring in the biosphere as suggested by (Watanabe 1994), then an estimated 7,000 species of fungi may exist in soil. In addition, although some soil fungi can be grown in culture, in many cases it is not possible to germinate resting structures such as spores, so that only vegetative mycelium is available for detailed analysis. Surveys of soil fungal diversity, which were popular during the 1960s and 1970s, have reappeared in the literature with the advent of DNA-based, culture-independent methods of analysis.

Culture-based estimates of soil fungal diversity require considerable effort and taxonomic expertise (Cosgrove et al. 2007). Culture-dependent approaches to characterize microbial communities additionally have built-in biases in the isolation of microorganisms. Recent attempts have been made to develop new culture media to maximize the recovery of diverse microbial groups (Davis et al. 2005; Vieira and Nahas 2005).

Culture-independent methods have recently been used in preference to traditional isolation techniques for microbial community analysis, including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), or temporal temperature gel electrophoresis (TTGE) methods (Muyzer et al. 1993; Torsvik et al. 1998; Muyzer 1999; Kirk et al. 2004; Kostanjsek et al. 2005). These techniques have proved highly successful in detecting soil microbial composition and diversity, thus providing insight into the response of soil ecosystems to environmental changes or anthropogenic disturbance. The combination of broad spectrum polymerase chain reaction (PCR) detection, coupled with single-strand conformation polymorphisms (SSCP) or DGGE, can provide more accurate answers to fundamental questions regarding ecosystem diversity. This technique does not, however, distinguish between active and resting stages (Bridge and Spooner 2001).

The most commonly isolated culturable soil fungi having significant ecological roles and functions belong to Glomeromycota, Zygomycota, Ascomycota, and Basidiomycota. Mycorrhizae and sugar fungi (zygomycetes) have been widely studied. The Ascomycota are the largest group in terms of number of species (approximately 33,000 plus another 16,000 known only as asexual forms), which span a range of nutritional modes from parasitic and pathogenic to plants, animals, and other fungi, through mutualists and saprotrophs (Kirk et al. 2001). A separate artificial phylum, Deutromycota or Fungi Imperfecti, has been devised for those fungi that lack known sexual reproduction, but the majority comprise asexual relatives of Ascomycota (Seifert and Gams 2001). The most familiar and economically important molds, including *Aspergillus* and *Penicillium,* are asexual forms of Ascomycota. Key literature in the identification of these groups of fungi appear in Gilman (2001), Ellis (1976), Domsch et al. (1993), Klich (2002), Mueller et al. (2004).

Some 20 functions of fungi were described by Christensen (1989), one of the main functions of fungi in soil being primary degraders. Many soil fungi have other roles and interactions, one of the most widely studied being mycorrhizal processes. Mycorrhizal relationships vary widely and may involve direct cross-feeding with plants, aiding in plant seed germination or the prevention of invasion by pathogens through niche exclusion (Brundrett et al. 1996).

Fungi are chemoheterotrophic organisms and are ubiquitous in subaerial and subsoil environments and are considered important decomposers and mutualistic symbionts of animal and plants. They are also pathogens and contribute to spoilage of natural and manufactured materials (Gadd 1993, 1999, 2006; Burford et al. 2003). Fungi also play an important role in the maintenance of soil structure due to their filamentous branching growth habit and exopolymer production. A fungal role in biogeochemical cycling of elements (e.g. carbon, nitrogen, phosphorus, sulfur, and metals) is well known and interlinked with the ability to adopt a variety of growth, metabolic and morphological strategies, their adaptive capabilities to environmental extremes, and their mutualistic associations with animals, plants, algae, and cyanobacteria (Burford et al. 2003; Gadd 2004; Braissant et al. 2004; Fomina et al. 2005a). Free-living fungi also have major roles in the decomposition of plant and other organic materials including cellulose, lignin, and chitin as well as the degradation of xenobiotics and the solubilization of minerals (Gadd 2004, 2005, 2006).

13.3 Heavy Metal Pollution in Water and Soil

Many researchers have reported heavy metal pollution in soil, especially in agricultural lands in different parts of the world (Sun et al. 2009; Fabiani et al. 2009; Yang et al. 2009; Nas et al. 2009). Fossil fuel combustion, mineral mining and processing, and the generation of industrial effluents and sludges, biocides and preservatives release a variety of toxic metal species into aquatic and terrestrial ecosystems, and this can have significant effects on biota (Gadd and Griffiths 1978; Gadd 1992a, 2000c, 2005, 2007b; Wainwright and Gadd 1997; Pokrovsky et al. 2008; Fabiani et al. 2009). Metal-rich habitats also occur due to natural localized ores and mineral deposits, and the weathering of rocks, minerals, soil, and sediments are a vast reservoir of metals. Restoration of metal-polluted habitats requires a functional microbial community for plant community establishment, soil development, and biogeochemical cycling.

Heavy metals are elements having a density over 5 g/cm^3. They are nondegradable and exist in number of inorganic and organic forms. Some heavy metals such as Fe, Cu, and Zn are essential trace elements but others, such as Cd and Pb, have no beneficial biological function and are toxic even in very small amounts. Cadmium, Pb, and Hg are regarded as the most toxic of the heavy metals. Another elemental toxicant, arsenic (As), is sometimes regarded as a heavy metal, although strictly speaking, it is a metalloid.

Contamination of soil and water by heavy metals has significant relevance, because metals cannot be degraded like most organic pollutants and they accumulate in terrestrial, aquatic and marine food chains (Smejkalova et al. 2003; Ortega-Larrocea et al. 2007). Metals such as Cd, Cr, Pb, Hg, As, copper (Cu), zinc (Zn), and nickel (Ni) are continuously being added to soils through agricultural activities such as long-term application of urban sewage sludge, and industrial activities such as waste disposal, waste incineration, and through vehicle exhausts. These sources

cause the accumulation of metals and metalloids in soils and pose threats to food safety and public health due to soil-to-plant transfer of metals.

Metals cause detrimental effects on both aquatic and terrestrial ecosystems and human health due to their mobilities and solubilities which determine their speciation (Kabata-Pendias 1992; Del Val et al. 1999). In some cases, soil may be contaminated to such an extent that it may be classified as a hazardous waste (Berti and Jacob 1996). Soil contamination with heavy metal mixtures is receiving increasing attention from the public as well as governmental agencies, particularly in developing countries (Yanez et al. 2002; Khan 2005).

Cadmium (Cd) is ubiquitous in the environment and has been recognized as one of the most hazardous of the heavy metal pollutants (Robards and Worsfold 1991; Christine 1997). Cadmium readily transfers from soil to food plants through root absorption and accumulates in tissues (Oliver 1997; Ortega-Larrocea et al. 2007), thereby potentially affecting human health (Adriano 1986; Smith 1996; Jose et al. 2002; Yao et al. 2003).

Cadmium concentrations ranging from 0 to 2.6 μg/L in drinking and natural waters have been reported from different parts of the world (Rajaratnam et al. 2002; Ho et al. 2003; Rosborg et al. 2003; Barton 2005; Virkutyte and Sillanpää, 2006; Asante et al. 2007). Natural Cd concentrations in water bodies rarely exceed the WHO guideline value of 3 μg/L (WHO 2006). High Cd concentrations in aquatic ecosystems are often reported in the surroundings of abandoned and active mines and metal smelters (Appleton et al. 2001; Miller et al. 2004; Lee et al. 2005a, b), especially where nonferrous metals are extracted (Florea et al. 2005). Phosphate fertilizers used in agriculture may also be enriched with Cd. Acidification of soil and water may release Cd bound to soil and sediments. Elevated Cd concentrations up to 57 μg/L (Seifert et al. 2000; Rajaratnam et al. 2002; Barton 2005) originating from soldered joints and zinc galvanized plumbing have been reported in tap water when first draw waters were studied. However, most of the households studied usually had acceptable Cd levels; for example, in Germany, Cd concentrations have been reported to exceed the WHO guideline value of 3 μg/L in only 0.7% of samples examined (Seifert et al. 2000).

In natural waters, Cd is distributed into three different fractions: dissolved, bound to suspended particulate matter (SPM), and precipitated/sedimented forms. Cadmium has a strong affinity for particulate matter, and sediments may contain over 100 mg/kg (Appleton et al. 2001; Woo and Choi 2001). As a result, soluble Cd concentrations in water are generally low, although the dissolved Cd fraction may be increased by high concentrations of dissolved organic matter (mainly fulvic and humic acids) (Linnik 2003) and low pH.

Excessive chromium (Cr) is present in the natural environment due to chrome plating and polishing operations, inorganic chemical production, cooling tower and steel mill effluents, and activities at wood-preserving facilities and petroleum refineries (USEPA 1990; Allen et al. 1998).

Chromium wastes pose a serious threat to public health and the environment. The chemical form of chromium (e.g., trivalent versus hexavalent) determines its toxicity, its mobility in the environment, and its availability to microorganisms.

Chromium(VI) has been shown to have carcinogenic, mutagenic and allergenic effects in humans and animals. In contrast, Cr(III) is considered a trace element essential for living systems (Costa 1997; Nies 1999). The toxicity of hexavalent chromium is from 100 to 1,000 times greater than that for the trivalent species (Onta and Hattori 1983; Wyszkowska et al. 2001). Chromium(VI) is toxic to biological systems due to its strong oxidizing potential that can damage cells (Kotas and Stasicka 2000). Within living cells, Cr(VI) complexes with organic compounds, interfering with metalloenzyme systems at high concentrations (Kotas and Stasicka 2000).

Lead contamination from anthropogenic sources is relatively common and high Pb concentrations have been detected in proximity to metal mines and smelters (Miller et al. 2004; Florea et al. 2005; Lee et al. 2005b). Point contamination on a smaller scale also occurs as a result of industrial emissions, agricultural practices, and improper disposal of sewage sludge. Before the introduction of unleaded fuels, the use of leaded gasoline was one of the major sources of Pb pollution to soil. Lead is also released into tap water from pipes, solders, and fittings of old plumbing systems (which may contain up to 50% Pb), and Pb concentrations up to 5,580 μg/L have been detected in tap water (Murphy 1993; Gulson et al. 1994; Gulson et al. 1997; Seifert et al. 2000; Barton et al. 2002; Rajaratnam et al. 2002; Fertmann et al. 2004).

The World Health Organization (WHO 2006) has established a limit of 10 μg/L for Pb in drinking water. Natural Pb concentrations of water bodies are generally low; background concentrations of <0.45–14 μg/L in groundwater have been reported (Smedley et al. 2002). Like Cd, Pb in water is distributed into three different fractions: dissolved, bound to SPM, and precipitated/sedimented. Lead has a strong affinity for particulate matter and, therefore, is mainly present in SPM and sediment fractions, but the dissolved amount is low (Balls 1988; Zarazua et al. 2006). However, Pb bound to SPM and sediments is at least partly reversible and may, therefore, be released to the surrounding water under suitable conditions (Chrastný et al. 2006).

13.4 Metal–Fungi Interactions and Development of Metal Resistance/Tolerance

Metals influence soil fungi by various means; for example, they can diminish total populations, impoverish fungal diversity, alter fungal morphology and physiological activity, and affect growth rate, reproduction processes, and enzyme production (Gadd 1992b, 1993; Martino et al. 2000). The response of *Penicillium* to heavy metals varies over a wide range of concentrations. Both sensitive and extremely resistant fungi of this genus have been reported in the literature. *Penicillium ochrochloron* is reported to grow in a saturated solution of copper sulfate (Stokes and Lindsay 1979). Metal effects vary not only among species and strains of fungi but also among different vegetative and reproductive forms of the same organism (Sabie and Gadd 1990). Fungal survival in the presence of toxic metals depends primarily on intrinsic

biochemical and structural properties, physiological and/or genetic adaptation including morphological changes, and environmental modification of metal speciation, and metal availability and toxicity. The relative importance of each of these is often difficult to determine (Gadd and Griffiths 1978; Gadd 1990, 1992b).

Heavy metal resistance in fungi has been investigated in detail in mutants isolated in the laboratory (Mohan and Sastry 1983) by gradual adaptation on toxic metal ion-containing media or by mutagenesis. A number of metal-resistant fungi isolated from polluted environments have also been reported (Ashida 1965; Gadd 1993; Zafar et al. 2007; Ahmad et al. 2006; Imran 2010); however, the mechanism of resistance in most cases was not studied. Resistance to heavy metals in fungi may be due to either of two mechanisms (1) transport blocks that restrict the entry of toxic metals into the cell and (2) intracellular sequestration into vacuoles or binding to specific proteins, viz. metallothioneins as described by Rao et al. (1997).

Numerous methods have been employed to determine metal tolerance in fungi. In vitro assays include sensitivity to spore germination, mycelia growth extension, and biomass production in the presence of various concentrations of metal salts in liquid and/or solid nutrient medium. These assays have demonstrated a range of levels of tolerance to different metals.

Metal-contaminated soil and wastewater harbor relatively more resistant fungal flora compared with noncontaminated media (Zafar et al. 2007; Ahmad et al. 2006; Ansari and Malik 2010); however, no strict criteria exist for the designation of a particular fungus as metal resistant/tolerant or sensitive. The minimum inhibitory concentration (MIC) of a metal sufficiently higher than MIC50 may be considered as tolerant or resistant.

13.5 Mechanisms of Metal Resistance and Tolerance

Metals and their compounds interact with fungi in various ways depending on metal species, organism, and environment. In addition, fungal metabolic activity can influence metal speciation and mobility.

Metal toxicity is greatly affected by the chemical behavior of the particular metal species, which is often influenced by the physical and chemical properties of the local environment. Metals exert toxic effects in many ways; for example, they can block the functional groups of important biological molecules such as enzymes, displace or substitute for essential metal ions, cause disruption of cellular and organellar membranes, and interact with systems which normally protect against harmful effects of free radicals generated during normal metabolism (Gadd 1992b, 1993; Avery et al. 1996; Howlett and Avery 1997). Fungi possess numerous qualities that influence metal toxicity including the production of metal-binding proteins (e.g., constituents of fungal cell walls [chitin, melanin] have significant metal binding abilities (Gadd and Griffiths 1978; Gadd 1993), organic and inorganic precipitation of metals, and active transport and intracellular compartmentalization. All these mechanisms are highly dependent on the metabolic and nutritional status

of the organism, as these will affect the expression of energy-dependent resistance mechanisms as well as synthesis of wall structural components, pigments, and metabolites, which affect metal availability and organism response (Gadd 1992b, 1993; Ramsay et al. 1999).

Fungi restrict entry of toxic metal species into cells by (1) reduced metal uptake and/or increased metal efflux; (2) metal immobilization, e.g., cell wall adsorption, extracellular precipitation of secondary neo-formed minerals (e.g. oxalates); and (3) extracellular metal sequestration by exopolysaccharides and other extracellular metabolites (Gadd 1993, 2001a, b, c; Macreadie et al. 1994; Blaudez et al. 2000; Perotto and Martino 2001; Baldrian 2003).

Metal-tolerant fungi survive in metal-enriched environments in part due to their abilities of intracellular chelation by the generation of metallothioneins and phytochelatins, and metal localization/sequestration within vacuoles. Fungal vacuoles play an important role in the regulation of cytosolic metal ion concentrations and the detoxification of potentially toxic metals (White and Gadd 1986; Gadd 1993; Gharieb and Gadd 1998; Liu and Culotta 1999). Metals preferentially sequestered by the vacuole include Mn^{2+} (Okorokov et al. 1985; Gadd and Laurence 1996), Fe^{2+} (Bode et al. 1995), Zn^{2+} (White and Gadd 1987), Co^{2+} (White and Gadd 1986), Ca^{2+} and Sr^{2+} (Okorokov et al. 1985; Borst-Pauwels 1989; Gadd 1993; Okorokov 1994), Ni^{2+} (Joho et al. 1995), and the monovalents K^+, Li^+, and Cs^+ (Okorokov et al. 1980; Perkins and Gadd 1993a, b). Recently, other researchers have discussed the mechanisms of metal resistance in soil fungi (Meharg 2003; Bučková et al. 2007; Gonçalves et al. 2007; Richie et al. 2007; Xiao et al. 2008).

13.5.1 Metal Solubilization

Solubilization of metal compounds is an important but unappreciated aspect of fungal physiology for the release of anions such as phosphate and essential metal cations into forms available for intracellular uptake, and transport through biogeochemical cycles. Fungal solubilization of metal compounds, including certain oxides, phosphates, sulfides and mineral ores, occurs by several mechanisms including (1) protonation of the anion of the metal compound, thereby decreasing its availability to the cation with the proton-translocating ATPase of the plasma membrane (production of organic acids being the source of protons) and (2) siderophore production (Gadd 1993, 1999; Sayer et al. 1995).

Organic acid anions are frequently capable of soluble complex formation with metal cations, thereby increasing mobility of the latter (White et al. 1997). Such complexation is dependent on relative concentrations of the anions and metals in solution, pH, and the stability constants of the various complexes (Denevre et al. 1996). A further mechanism of metal solubilization is the production of low-molecular-weight iron-chelating siderophores, which solubilize Fe^{3+}. Siderophores are the most common means of acquisition of Fe by bacteria and fungi and are effective over a wide range of soils, including calcareous soil. The most common fungal siderophore is ferrichrome (Crichton 1991).

Acidification of soil can lead to metal release via a number of obvious routes, e.g., competition between protons and metal in a metal–anion complex or in a sorbed form, resulting in the release of free-metal cations. Heterotrophic metabolism can also lead to leaching as a result of the efflux of organic acids and siderophores. Organic acids supply both protons and metal-complexing anions (Burgstaller and Schinner 1993; Gadd 1999; Gadd and Sayer 2000). Citrate and oxalate anions can form stable complexes with a large number of metals. Many metal citrates are highly mobile and not readily degraded (Francis et al. 1992). Oxalic acid can also act as a leaching agent for those metals that form soluble oxalate complexes, including Al and Fe (Strasser et al. 1994).

In many fungi, an important leaching mechanism occurs through the production of organic acids (e.g., oxalic acid and citric acid) (Adams et al. 1992; Francis et al. 1992; Denevre et al. 1996; Sayer et al. 1997; Gadd 1999, 2000a; Sayer and Gadd 2001; Jarosz-Wilkolazka and Gadd 2003; Fomina et al. 2005a; Ansari 2004; Imran 2010)

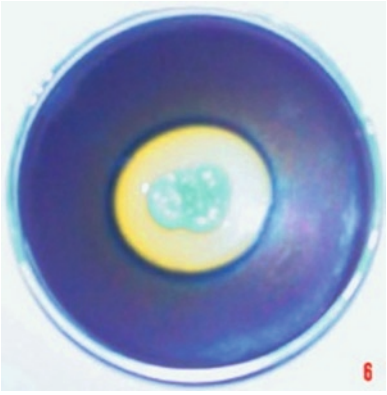

Fig. 13.1 Organic acid production by *Penicillium spp.* in plate (Ansari 2004)

Fig. 13.2 Organic acid production by *Aspergillus spp.* in plate (Ansari 2004)

(Figs. 13.1 and 13.2). Organic acid excretion by fungi is both inter- and intra-specific and can be strongly influenced by the presence of toxic metals (Sayer et al. 1995; Sayer and Gadd 2001; Fomina et al. 2004, 2005c).

13.5.2 Metal Immobilization

Toxic metal species including radionuclides can be bound, accumulated, and precipitated by fungi. Fungal biomass can act as a metal sink by (1) metal biosorption to biomass cell walls, pigments, and extracellular polysaccharides; (2) intracellular accumulation and sequestration (including uptake with complexation to ligands such as sulfur-containing peptides [e.g. metallothioneins] [Gadd 1993; Sarret et al. 1998, 2002; Fomina et al. 2005b]); or (3) precipitation of metal compounds onto and/or around hyphae. Some fungi can precipitate metals in amorphous and crystalline forms, such as oxalates and other secondary mycogenic minerals (Gadd 1999; Burford et al. 2003, 2006).

In addition to immobilizing metals, the above processes reduce the external free metal concentration and drive the equilibria to release more metal ions into soil solution (Gadd 1993, 2000a, b; Sterflinger 2000). Fungi can be highly efficient accumulators of soluble and particulate forms of metals (e.g., Ni, Zn, Ag, Cu, Cd, and Pb), especially from dilute external concentrations (Gadd 1993, 2000a, b, 2001b, c; Baldrian 2003).

13.5.3 Metal Transformations

Metal transformation embraces the mechanisms by which fungi (and other microorganisms) effect changes in metal speciation and mobility. Transformations are essential components of biogeochemical cycles for metals as well as all other elements including carbon, nitrogen, sulfur, and phosphorus. Fungi and other microorganisms transform metals via oxidation, reduction, methylation, and dealkylation (Gadd 1992a). Some enzymatic metal transformations may be involved in survival since certain transformed metal species are less toxic and/or more volatile than the original species. Reductions carried out by fungi include Ag^{+} to metallic Ag^{0} which is deposited in and around cells (Kierans et al. 1991) and Cu^{2+} to Cu^{+} by cell wall-associated compounds in *Debaryomyces hansenii* (Wakatsuki et al. 1988, 1991; Breuer and Harms 2006).

13.6 Biosorption

Biosorption is the process by which metals are sorbed or complexed to either living or dead biomass (Volesky and Holan 1995). Binding of metal ions onto cell walls and other external surfaces in fungal biomass (Gadd 1990, 1993;

Sterflinger 2000) can be an important passive process in concentrating metals in soils and contaminated aquifers (McLean et al. 1996; Berthelin et al. 1995). It has been suggested that stimulating the growth of indigenous microorganisms with metal biosorptive capacities may be a useful strategy for immobilizing metals in soils and preventing contamination of underlying groundwater supplies (Valentine et al. 1996). Furthermore, it is possible to envision a barrier of microorganisms with biosorptive abilities established in subsurface environments to remove metals from groundwater flowing through. Although small-scale bioremediation of mine drainage with biosorption has been documented (Ledin and Pedersen 1996), biosorption has been evaluated primarily as a strategy for removing metals from waste streams. Biosorption may be economically competitive with ion exchange or chemical precipitation for treating some waste streams (Eccles 1995). One strategy to enhance the applicability of biosorption over alternative techniques for metal removal is to survey for novel microorganisms with unique biosorption capacities (Hu et al. 1996; Vesper et al. 1996).

13.6.1 Biosorbents

Adsorptive removal of heavy metals from aqueous effluents, which has received much attention in recent years, is usually achieved using activated carbon or activated alumina (Faust and Aly 1987; Ouki et al. 1997; Hsisheng and Chien-To 1998; Ali et al. 1998; Ralph et al. 1999; Shim et al. 2001; Monser and Adhoun 2002; Igwe and Abia 2005).

Certain biosorbents bind and collect a wide range of heavy metals with no specificity, whereas others are specific for certain types of metals (Hosea et al. 1986; Volesky and Kuyucak 1988). When choosing biomass for metal biosorption experiments, its origin must be taken into account. Biomass can originate from

1. Industrial wastes, which should be obtained free of charge,
2. Organisms readily available in large quantities in nature, and
3. Organisms experiencing rapid growth and cultivated or propagated for biosorption purposes.

Biosorbents prepared from naturally abundant biomass are primarily of algae, fungi, moss, or bacteria that have been killed by washing with acids or bases or both, before drying and granulation (Brierley 1990; Kratochvil et al. 1997). Living or dead fungal biomass and fungal metabolites have been used to remove metal or metalloid species, metal compounds and particulates, radionuclides and organometal compounds from solution by biosorption (Gadd and White 1989, 1990, 1992, 1993; Wang and Chen 2006). These processes are ideally suited for use in bioreactors (Gadd 2000a).

13.6.2 Metal Binding to Cell Walls

The wall is the first cellular site of interaction with external metal species. Metal removal from solution may be rapid, although rates depend on numerous factors such as type of metal ion and biomass, metal concentration and environmental factors such as pH, oxidation–reduction status, presence of competing ions, etc. Metabolism-independent modes of metal binding to fungal walls include ion exchange, adsorption, complexation, precipitation, and crystallization (Mullen et al. 1992).

The fungal cell wall is composed primarily of polysaccharides, some of which may have associated proteins with other components including lipids and melanins. The specific components of the fungal cell wall include the following.

13.6.2.1 Skeletal Elements

Chitin: β-1-4-linked homopolymer of *N*-acetyl-D-glucosamine
β-Glucans: β-1-3-glucan homopolymer composed of D-glucose units with β-1-3- and β-1,6-glucosidic bonds (R-glucan)
Cellulose: β-1,4-linked homopolymer of glucose

13.6.2.2 Matrix Components

α-Glucan: α-1,3-homopolymer of glucose (s-glucan)
α-1,3- and α-1,4-Linked glucan (nigeran)
Glycoproteins
Mannoproteins

13.6.2.3 Miscellaneous Components

Chitosan: β-1,4-polymer of D-glucosamine
D-galactosamine polymers
Polyuronides
Melanins
Lipids

The fungal cell wall thus has important protective properties and so may act as a barrier controlling uptake of solutes, including potentially toxic metal species, into the cell (Gadd and Griffiths 1978; Gadd 1986a, b; Ono et al. 1988) and also indirectly affects the intracellular ionic composition by restricting cellular water.

13.6.3 Transport of Toxic Metal Cations

Most research on metal ion transport in fungi has concerned K^+ and Ca^{2+}, largely because of their importance in fungal growth, metabolism, and differentiation. The transport of toxic metal species is still poorly understood.

Transport systems in cell membranes are usually classified as either carrier or channel systems. In the carrier system, conformational changes in the transport protein are believed to result in alternate exposure of the transport binding site on each side of the membrane. Carriers include all metabolically coupled and H^+-gradient-driven transport systems. Fluxes through such systems saturate with respect to ligand concentration and, if a current is carried, with respect to membrane potential (Sanders 1990).

Ion channels are a class of protein that function as gated pores in the plasma membrane allowing the flow of ions down electrical and/or chemical gradients (Gustin et al. 1986). Channels have higher turnover rates than carriers, 10^{7-8} s^{-1} compared with 10^{2-5} s^{-1}, respectively (Sanders 1990).

13.6.4 Metal Uptake by Living Cells

Penicillium is known to remove a variety of heavy metals from aqueous solution. Spores of *Penicillium italicum* were shown to accumulate Cu (Somers 1963; Kapoor and Viraraghavan 1995). Metal accumulation by growing cells varied with age of the cell. Maximum metal uptake occurred during the lag period, or the early stages of growth and declined as cultures reached a stationery phase. *A. niger*, *P. spinolosum*, and *Trichoderma viride* showed a similar uptake pattern (Townsley and Ross 1985, 1986; Kapoor and Viraraghavan 1995). Other, researchers have also reported metal uptake by living cells (Bayramoglu et al. 2006; Zafar et al. 2007; Melgar et al. 2007; Akhtar et al. 2007; Pakshirajan and Swaminathan 2009).

The uptake of metals by living cells depends on contact time, solution pH, culture conditions, initial metal ion concentration, and the concentration of cells in aqueous solution (Kurek et al. 1982; Galun et al. 1987; Siegel et al. 1987). Huang et al. (1988) observed that Cd biosorption on various fungal strains was pH-sensitive. *Aspergillus oryzae*, *Fusarium solani*, and *Candida utilis* were found to take up higher concentrations of metal in the acidic range. *Mortierella ramannianc, Rhizopus sexualis*, *R. stolonifer*, *Zygorhynchus hetergamus*, *Z. moelleri*, *A. niger*, *Mucor recemosus*, *Penicillium chrysogenum*, and *T. viride* removed Cd from aqueous solutions (Azab et al. 1990; Kurek et al. 1982; Ross and Townsley 1986; Kapoor and Viraraghavan 1995).

13.6.5 Intracellular Fate of Toxic Metals

Both in laboratory and in field studies, it has been shown that the toxicity of a given metal depends on species and chemical properties as well as environmental factors

(e.g., adsorption to solid surfaces, complexation, or precipitation) (Hughes and Poole 1989; Gadd 1992a, b).

Toxic effects include ion displacement and/or substitution of essential ions from cellular sites and blocking of functional groups of important molecules, e.g., enzymes, polynucleotides, and essential transport systems. This results in denaturation and inactivation of enzymes and disruption of cell organelle membrane integrity.

Metal-binding proteins are important in the modulation of intracellular concentrations of both potentially toxic and essential metal ions. The superfamily of proteins called metallothioneins may achieve these by binding the metal ion to cysteine thiolate groups (Hamer 1986).

Polypeptides are designated as "metallothioneins," if they possess specific properties including low molecular mass, high metal content, high cysteine (cys) content, abundant cys-*x*-cys sequences (where *x* is an amino acid other than cys), and metal-thiolate clusters, and lack the aromatic amino acids and histidine.

The following subdivision of metallothioneins into three classes has been recommended (Rauser 1990).

Class I: Polypeptides with locations of cysteine closely related to those in equine renal metallothionein.

Class II: Polypeptides with locations of cysteine only distantly related to those in equine renal metallothionein.

Class III: Typical, nontranslationally synthesized metal thiolate polypeptides.

13.6.6 Metal Transformations Within Fungi

Microbes play key geoactive roles in the biosphere, particularly in the areas of element biotransformations and biogeochemical cycling, metal and mineral transformations, decomposition, bioweathering, and soil and sediment formation. Numerous categorie of microbes, including prokaryotes and eukaryotes, and their symbiotic associations with each other and "higher organisms," can contribute actively to geological phenomena, and central to many such geomicrobial processes are transformations of metals and minerals. Fungi possess a variety of properties that can effect changes in metal speciation, toxicity, and mobility, as well as mineral formation or mineral dissolution or deterioration. Such mechanisms are important components of natural biogeochemical cycles for metals as well as associated elements in biomass, soil, rocks, and minerals, e.g., sulfur and phosphorus, and metalloids, actinides, and metal radionuclides (Gadd 2010).

Fungi are intimately involved in biogeochemical transformations at local and global scales, and although such transformations occur in both aquatic and terrestrial habitats, it is the latter environment where fungi probably have the greatest influence. Within terrestrial aerobic ecosystems, fungi may exert an especially profound influence on biogeochemical processes, particularly when considering soil, rock and mineral surfaces, and the plant root–soil interface. The geochemical transformations that take place can influence plant productivity and the mobility of toxic elements and

substances and are therefore of considerable socioeconomic relevance, including human health. Of special significance are the mutualistic symbioses, lichens, and mycorrhizas. Some fungal transformations have beneficial applications in environmental biotechnology, e.g., in metal leaching, recovery and detoxification, and xenobiotic and organic pollutant degradation. They may also cause adverse effects when these processes are associated with the degradation of foodstuffs, natural products, and building materials, including wood, stone, and concrete (Gadd 2007a).

13.6.7 Metal Sorption by Dead Cells

The biosorption capacity of dead cells may be greater to, equivalent to, or less than that of living cells. Recently, several researchers reported metal removal by dead microbial cells (Naeem et al. 2006; Chen and Wang 2007; Akhtar et al. 2007; Bishnoi et al. 2007; Tsekova et al. 2007).

Use of dead biomass in industrial applications offers certain advantages over that of living cells. Systems using living cells are likely to be more sensitive to metal-ion concentration (i.e., toxicity effects) and adverse operating conditions (pH and temperature). Furthermore, a constant, regulated nutrient supply is required for systems using living cells (with increased operating cost for the management of waste streams), and recovery of metal and regeneration of biosorbent are more complicated for living cells.

In such a biosorption system cells can be killed by physical treatment methods using heat (Siegel et al. 1987), autoclaving, and vacuum drying (Tobin et al. 1984; Huang et al. 1988), or chemicals such as acids, alkalis, and detergents (Tsezos and Volesky 1981; Ross and Townsley 1986; Huang et al. 1988; Rao et al. 1993; Kapoor and Viraraghavan 1995).

13.6.8 Mechanism of Biosorption

Veglio and Beolchini (1997) described biosorption mechanisms on the basis of cellular metabolism (i.e., metabolism-dependent and metabolism-independent) and according to location where the removed metal is located.

The process of biosorption may be classified as follows:

1. Extracellular accumulation/precipitation
2. Cell surface sorption/precipitation
3. Intracellular accumulation

13.6.8.1 Extracellular Accumulation/Precipitation

Some prokaryotic (bacteria and archaea) and eukaryotic (algae and fungi) microorganisms produce or excrete extracellular polymeric substances (EPS) such as polysaccharides,

glycoprotein, lipopolysaccharides, soluble peptides, etc. These substances possess a substantial quantity of anionic functional groups which can adsorb metal ions. Research published on metal biosorption with EPS focuses mainly on the bacteria, such as *Bacillus megaterium*, *Acinetobacter, Pseudomonas aeruginosa,* sulfate-reducing bacteria (SRB), cyanobateria, or activated sludge (Liu et al. 2001), whereas EPS studies for fungi and algae are limited (Wang and Yang 1996); Flemming and Wingender (2001) discovered that the initial rate of Pb^{2+} uptake by live cells of *Saccharomyces cerevisiae* is lower than that of dead cells, while in the case of *A. pullulans*, both the capacity and the initial rate of Pb^{2+} accumulation in live cells are greater than those in dead cells. This result was due to the presence of EPS in live *A. pullulans*.

13.6.8.2 Cell Surface Sorption/Precipitation

Numerous chemical groups have been suggested to contribute to metal biosorption either by whole organisms such as algae (Crist et al. 1981; Greene et al. 1987) and bacteria (Brierley 1990; Mann 1990) or by molecules such as biopolymers (Hunt 1986; Macaskie and Dean 1990). These groups comprise hydroxyl, carbonyl, carboxyl, sulfhydryl, thioether, sulfonate, amine, imine, amide, imidazole, phosphonate, and phosphodiester groups. The importance of any given group for biosorption of a particular metal by particular biomass depends on factors including:

1. The number of sites occurring within the biosorbent material
2. The accessibility of the sites
3. The chemical state of the site (i.e. availability)
4. Affinity between site and metal (i.e. binding strength)

The cell wall consists of a variety of polysaccharides and proteins and hence offers a number of active sites capable of binding metal ions (Kuyucak and Volesky, 1989). Thus, it is regarded as a complex ion exchanger, similar to a commercial resin. Differences in cell wall composition among different groups of microorganisms, viz. algae, bacteria, cyanobacteria and fungi, and intra group differences can thus result in significant differences in type and amount of metal ion binding (Horikoshi et al. 1981; Friis and Myers-Keith 1986; Muraleedharan et al. 1991). The various groups involved in metal binding have been discerned by modification/blocking of the groups (Tobin et al. 1990).

The cell wall tends to be the first cellular structure to come into contact with metal ions, excluding a possible extracellular layer mainly related to bacterial cells. Two basic mechanisms of metal uptake by the cell wall are (1) stoichiometric interaction between functional groups of the cell wall including phosphate, carboxyl, and amine as well as phosphodiester and (2) physicochemical inorganic deposition via adsorption or inorganic precipitation.

Other mechanisms such as complexation, ion exchange, adsorption (by electrostatic interaction or van der Waals force), inorganic microprecipitation, oxidation, and/or reduction have been proposed to explain metal sorption by organisms (Volesky 1990a, b; Liu et al. 2002).

Kapoor and Viraraghavan (1997) showed that in dried fungal biomass of *A. niger*, amine and carboxyl groups were important functional groups involved in Pb, Cd, and Cu biosorption, and they reported that phosphate groups and the lipid fraction of the biomass did not play a significant role in biosorption of the metal ions studied.

Brady and Tobin (1995) found that the total metal ions displaced accounted for only a small portion of the metal ions taken up in the biosorption of metal ions by freeze-dried *R. arrhizus***.** This indicates that ion exchange is neither the sole nor the main mechanism for metal biosorption by fungi. However, Davis et al. (2003) believed ion exchange was the main mechanism for metal ion uptake by brown algae.

Precipitation and redox reactions of heavy metals on the cell surface are also reported by many researchers. A research group at Xiamen University in China found that precious metal ions such as Pd^{2+} (Liu et al. 2003; Xie et al. 2003a), Pt^{4+} (Xie et al. 2003b), Au^{3+} (Lin et al. 2005), Ag^{+} (Lin et al. 2001), and Rh^{3+} (Lin et al. 2001) were unexceptionally bound to the cell wall of yeast and then reduced in situ to the corresponding solids.

Biosorption of heavy metals often involves many mechanisms. Kratochvil et al. (1998) proved that the maximal uptake of Cr^{6+} by protonated *Sargassum* biomass at pH 2 was due to simultaneous anion exchange and the reduction of Cr^{6+} to Cr^{3+}.

13.6.8.3 Intracellular Accumulation/Precipitation

Metal transport across the cell membrane results in intracellular accumulation, which is dependent on cellular metabolism. This implies that this mode of biosorption may take place only within viable cells (Veglio and Beolchini 1997).

After entering the cell, metal ions are compartmentalized into different subcellular organelles (e.g. mitochondria and vacuoles). Vijver et al. (2004) summarized metal ion accumulation strategies, in particular the internal compartmentalization strategies. The mechanism primarily relates to the presence of low-molecular-weight metal-binding proteins, the metallothioneins (MT), which are cysteine-rich and occur in the animal kingdom, plants, eukaryotic microorganisms, and some prokaryotes. MT can be induced by many substances, including heavy metal ions such as Cd, Cu, Hg, Co, and Zn (Vijver et al. 2004).

In addition to MT, other cellular thiols influencing the sensitivity to toxic metals include glutathione (GSH), phytochelatins (β cadystins (α-Glu-Cys) nGly), and labile sulfide (Perego and Howell 1997; Gharieb and Gadd 2004). Tripeptide glutathione (GSH) is a typical low-molecular-weight cellular thiol and functions as a storage form of endogenous sulfur and nitrogen as well as for detoxification of metal ions. GSH in *S. cerevisiae* may account for 1% of cell dry weight (Gharieb and Gadd 2004). The role of the vacuole in the detoxification of metal ions was investigated by Ramsay and Gadd (1997), who showed that a vacuole-deficient strain displayed much higher sensitivity and a lower biosorption capacity for Zn, Mn, Co, and Ni.

Many genes involved in the tolerance to uptake or detoxification of metal ions have been identified (Rosen 2002). For example, the *S. cerevisiae* Arr4p plays an important role in the tolerance to As^{3+}, As^{5+}, Co^{2+}, Cr^{3+}, Cu^{2+}, and VO_4^{3-} (Shen et al. 2003). Genetic technologies, including cell surface display technologies have been applied to improve the performance of biomass in metal removal from solution (Bae et al. 2003; Kuroda et al. 2002; Wang 2005). Kuroda et al. (2002) constructed a cell surface-modified yeast *S. cerevisiae*, which produces histidine hexapeptide. This engineered yeast can chelate Cu ion and possesses the property of self-aggregation, which indicates the potential application for bioremediation of heavy metal pollution.

13.6.9 Factors Affecting Heavy Metal Biosorption

Bioremoval of a heavy metal using microorganisms is affected by several factors, including the specific surface properties of the microorganism and the physicochemical parameters of the solution such as temperature, pH, metal ion concentration, metal solubility, metal valence, concentration of complexing agents, and particle size (Brown and Lester 1979). Butter et al. (1998) showed temperature variations from 15 to 35°C did not affect Cd sorption by dead *Streptomyces* biomass. Also, Kasan (1993) found that the complexation/removal of Cr, Pb, and Zn by living activated sludge was independent of temperature.

Several studies are reported in the literature which have investigated the effect of pH on biosorption of metals. Most investigators have reported negligible metal sorption at pH values <4.0 (Tien and Huang 1987; Delgado et al. 1998; Wang et al. 1999). These results could be explained by the competition between hydrogen ions and metal ions for the sorption sites of cells. At very low pH values, metal cations and protons compete for binding sites on cell walls, which results in lower metal uptake. Biosorbent concentration has also been shown to be an important factor in the biosorption process. Metal uptake increased when biomass concentration decreased (Esposito et al. 2001); as an increase in biomass concentration leads to interference between binding sites (Veglio et al. 1997; Esposito et al. 2001).

Another factor affecting biosorption is initial metal concentration. It has been reported that adsorption rate increases with increasing initial metal concentration. For example, adsorption of Fe^{2+}, Pb^{2+}, and Cd^{2+} by *S. leibleini* increased with increasing initial metal ion concentrations up to 150 mg/L. At high concentrations, the adsorption rates did not change (Ozer et al. 1999).

13.6.9.1 Biomass Pretreatment Effect on Biosorption

Living cells have been pretreated using physical and chemical methods to increase metal biosorption capacity. Physical pretreatment methods have included heat treatment, autoclaving, freeze-drying, and boiling. Chemical pretreatment methods such

as reacting cells, especially fungal cells, with acids, alkaline and organic chemicals have been reported (Wase and Forster 1997; Kapoor and Viraraghavan 1998; Zhao and Duncan 1998).

Yan and Viraraghavan (2000) studied the effect of pretreatment of *Mucor rouxii* biomass on bioadsorption of Pb^{2+}, Cd^{2+}, Ni^{2+}, and Zn^{2+}. Pretreatment with detergent and alkalis such as NaOH, Na_2CO_3, and $NaHCO_3$ improved or maintained the bioadsorption capacity in comparison with live *M. rouxii* biomass. Acid pretreatment using HCl, H_2SO_4, and $C_2H_4O_2$ resulted in a significant reduction in bioadsorption capacity. To improve the bioadsorption capacity for metal ions by dead biomass, alkali pretreatment was an effective method (Yan and Viraraghavan 2000).

Bai and Abraham (2002) reported that the treatment of the biosorbent with mild alkalies (0.01 N NaOH and ammonia solution) and formaldehyde (10% w/v) deteriorated biosorption efficiency. However, extraction of biomass powder in acids (0.1 N HCl and H_2SO_4), alcohols (50% v/v, CH_3OH and C_2H_5OH), and acetone (50% v/v) improved Cr uptake capacity. Reaction of cell wall amino groups with acetic anhydride reduced biosorption potential drastically. Blocking of COOH groups by treatment with water-soluble carbodiimide resulted in an initial lag in Cr binding. Biomass modification experiments conducted using cetyl trimethyl ammonium bromide (CTAB), polyethylenimine (PEI), and amino propyl trimethoxy silane (APTS) improved biosorption efficiency to exceptionally high levels.

13.7 Biosorption Potential of Fungal Biomass

Heavy metal biosorption potential of different treatment fungi are known and vary greatly. This variation is probably due to the different methods used and types of biomass and their pretreatment (Table 13.1). Heavy metal biosorption preference by various fungi in single/multimetal solutions is also variable as reported by various workers (Table 13.2).

Sorption of Pb by nonliving *P. chrysogenum* biomass was strongly affected by pH (Niu et al. 1993). Within a pH range of 4–5, the saturated sorption uptake of Pb^{2+} was 116 mg/g dry biomass, higher than that of activated charcoal and other microorganisms. At pH 4.5, *P. chrysogenum* biomass exhibited sorption preference for metals in the following order: $Pb > Cd > Cu > Zn > As$. Sorption of Pb^{2+} remained unchanged in the presence of Cu^{2+}, and As^{3+} decreased in the presence of Zn^{2+} and increased in the presence of Cd^{2+}. Volesky and May-Phillips (1995) found that living and nonliving biomass of *S. cerevisiae* differs in uptake of uranium (U), Zn, and Cu at the optimum pH of 4–5. Dead cells of *S. cerevisiae* removed approximately 40% more U or Zn than corresponding live cultures. The maximum Pb biosorption capacity at pH 6 of *M. rouxii* was estimated at 769 mg/g dry biomass, significantly higher than that of most microorganisms. Biomass of *M. rouxii* showed specific selectivity for Pb^{2+} over Zn^{2+}, Ni^{2+}, and Cu^{2+} (Lo et al. 1999). Bai and Abraham (2001) reported that the optimum pH for biosorption of Cr^{6+} was 2.0. Adsorption capacity of biomass increased with increasing concentration

Table 13.1 Heavy metal biosorption potential of different fungi

Biosorption capacity (mg/g)	Biosorbent	Treatment	Metals	References
0–10	*Mucor rouxii*	Immobilized	Pb, Cd, Ni, Zn	Yan and Viraraghavan (2001)
	Aspergillus foetidus		Cr	Prasanjit and Sumathi (2005)
	Aspergillus spp.		Cr, Cd	Zafar et al. (2007)
	Mucor rouxii	Dead	Ni, Zn	Yan and Viraraghavan (2003)
	Rhizopus spp	–	Cr, Cd	Zafar et al. (2007)
	S. cerevisiae	Immobilized	Pb	Zhang et al. (2009)
11–20	*Aspergillus flavus*	Dead	Pb, Cu	Akar and Tunali (2006)
	Mucor rouxii	Dead	Cd	Yan and Viraraghavan (2003)
	Penicillium chrysogenum		Ni	Tan and Cheng (2003)
	Penicillium spp	Dead	Cr, Ni, Cd	Ahmad et al. (2006)
	Aspergillus niger	Dead	Cr, Cd	Ahmad et al. (2006)
	Trichoderma viride	Immobilized	Cr	Bishnoi et al. (2007)
	Rhizopus oryzae	Living	Cu	Bhainsa and D'souza (2008)
	Aspergillus niger	Free	Cu	Tsekova et al. (2010)
21–30	*Mucor rouxii*	Dead	Pb	Yan and Viraraghavan (2003)
	Penicillium canescens		As	Say et al. (2003a)
	Penicillium chrysogenum		Cr, Zn	Tan and Cheng (2003)
	Aspergillus niger	Dead	Ni	Ahmad et al. (2006)
	Aspergillus niger		Cu	Mukhopadhyay et al. (2007)
	Rhizopus arrhizus	Dead/living	Cu	Subudhi and Kar (2008)
31–50	*Funalia trogii*	Immobilized (live)	Zn	Yakup et al. (2004)
	Penicillium canescens		Hg	Say et al. (2003b)
	Penicillium cyclopium		Cu	Ianis et al. (2006)
	Penicillium purpurogenum		Cr	Say et al. (2003b)
	Aspergillus niger	Immobilized	Cu	Tsekova et al. (2010)

(continued)

Table 13.1 (continued)

Biosorption capacity (mg/g)	Biosorbent	Treatment	Metals	References
51–100	*Funalia trogii*	Immobilized (heat inactivated)	Zn	Yakup et al. (2004)
	Phanerochaete chrysosporium	Resting cells	Ni, Pb	Ceribasi and Yetis (2001)
	Penicillium simplicissimum		Cd, Zn, Pb	Fan et al. (2008)
	Aspergillus niger	Free/immobilized	Cd	Tsekova et al. (2010)
100–200	*Funalia trogii*	Immobilized (live/heat inactivated)	Cd	Yakup et al. (2004)
	Penicillium canescens		Cd	Say et al. (2003b)
More than 200	*Funalia trogii*	Immobilized (live/heat inactivated)	Hg	Yakup et al. (2004)
	Penicillium canescens		Pd	Say et al. (2003b)

Table 13.2 Heavy metal biosorption preference by various fungi in single/multimetal solutions

pH	Biosorbent	Biosorption preference/order	References
0–2	*Aspergillus niger*	Cr^{6+}	Chhikara and Dhankhar (2008)
2–4	*Trichoderma viride* (immobilized)	Cr^{6+}	Bishnoi et al. (2007)
4–6	*Penicillium chrysogenum*	Pb > Cd > Cu > Zn > As	Niu et al. (1993)
	Sacchromyces cerevisiae	U > Zn > Cd > Cu	Volesky and May-Phillips (1995)
	Baker's yeast (nonliving)	Zn > Cd > U > Cu	Volesky and May-Phillips (1995)
	Baker's yeast (living)	Zn > Cu = Cd > U	Volesky and May-Phillips (1995)
	Mucor rouxii	Pb > Zn > Cd > Ni	Yan and Viraraghavan (2003)
	Aspergillus flavus (heat inactivated)	Pb > Cu	Akar and Tunali (2006)
	Phanerochaete chrsosporium	Cd > Cu	Pakshirajan and Swaminathan (2009)
6–8	*Mucor rouxii*	Pb > Zn > Cd > Ni	Yan and Viraraghavan (2003)
	Aspergillus niger	Ni	Amini et al. (2009)
	Funalia trogii (immobilized live)	Hg^{2+} > Cd > Zn	Yakup et al. (2004)
	Sacchromyces cerevisiae	Ni > Cd	Fereidouni et al. (2009)

of ions, temperature, and agitation speed, and optimum sorption capacity was determined at 45°C and 120 rpm.

Biosorption in the order Cu > Zn > Cd was observed in *Microcystis sp*. in single-, bi-, and trimetallic combinations. The order of inhibition of Cu, Zn, and Cd biosorption in bi- and trimetallic combinations suggested possible screening or competition of the binding sites on cell surfaces (Pradhan and Rai 2001). Yan and Viraraghavan (2001) studied the biosorption capacity of *M. rouxii* biomass and immobilized it in a polysulfone matrix. For single-component metal solutions, the metal removal capacities of the beads for Pb, Cd, Ni, and Zn were 4.06, 3.76, 0.36, and 1.36 mg/g, respectively. For a multicomponent metal solution, the capacities were 0.36, 0.31, and 0.40 mg/g for Cd, Ni, and Zn, respectively. Say et al. (2001) studied the biosorption of Cd^{2+}, Pb^{2+}, and Cu^{2+} from artificial wastewaters onto dry biomass of *Phanerochaete chryosporium* in the concentration range of 5–500 mg/L. Maximum absorption of metal ions on fungal biomass was obtained at pH 6.0. The experimental biosorption data for Cd^{2+}, Pb^{2+}, and Cu^{2+} ions were in good agreement with those calculated by the Langmuir model.

Immobilized mycelia of *Rhizopus delemar* showed an occasional increase in uptake compared with that of free cells. Metal ion accumulation from a mixed solution decreased slightly for Co and Fe and decreased considerably for Cu ions. Metal uptake was examined in immobilized column experiments; >92% heavy metal removal was achieved from a mixed solution during five cycles (Tsekova and Petrov 2002). Similarly, Yan and Viraraghavan (2003) reported that biosorption by dead biomass of *M. rouxii* was reduced in the presence of other metal ions but total biosorption capacity increased, indicating the capability of *M. rouxii* biomass in adsorbing multimetal ions. *A. niger* removed >98% Ni from a liquid medium after 100 h growth but did not remove other metals (Magyarosy et al. 2002).

Tan and Cheng (2003) used alkaline pretreatment of *P. chrysogenum* to remove proteins and nucleic acids from cells, thus increasing adsorption capacities from 18.6 to 27.2 mg/g for Cr^{3+}, from 13.2 to 19.2 mg/g for Ni^{2+}, and from 6.8 to 24.5 mg/g for Zn^{2+}. Yakup et al. (2004) measured maximum adsorption of metals on calcium alginate and both live and inactivated immobilized fungal preparations of *Funalia trogii* at pH 6.0. Metal biosorption capacities of heat inactivated-immobilized *F. trogii* for Hg^{2+}, Cd^{2+}, and Zn^{2+} were 403.2, 191.6, and 54.0 mg/g, respectively, while biosorption capacities of the immobilized live cells was 333.0, 164.8, and 42.1 mg/g, respectively. The same order of affinity on a molar basis was observed for single- or multi-metal ions.

Heat inactivated biomass of *Aspergillus flavus* showed maximum biosorption values of 13.5 mg/g for Pb^{2+} and 10.8 mg/g for Cu^{2+} at pH 5.0 with an equilibrium time of 2 h. The results indicated that *A. flavus* is a suitable biosorbent for removal of Pb^{2+} and Cu^{2+} ions from aqueous solution (Akar and Tunali 2006). Ahmad et al. (2006) reported that alkali-treated, dried, and powdered mycelia of metal-tolerant fungal isolates of *Aspergillus* and *Penicillium* have high biosorption capacities for Cr, Ni, and Cd. Biosorption of all metals was found to be higher at 4 mM initial metal concentration when compared with that at 2 and 6 mM. At 4 mM initial metal concentration, Cr biosorption was 18.1 and 19.3 mg/g of *Aspergillus* and *Penicillium* biomass, respectively. Similarly, biosorption of Cd and Ni ions was maximal at 4 mM initial metal concentration by *Aspergillus* (19.4 mg/g for Cd and 25.1 mg/g for Ni) and *Penicillium* (18.6 mg/g for Cd and 17.9 mg/g for Ni). Dried mycelial biomass of Co-resistant fungi belonging to *Mortierella* isolated from serpentine soil of Andaman (India) removed almost 50% of 4.0 mM Co from aqueous solution (Pal et al. 2006). The metal biosorption capacity of the isolate accelerated with increasing Co concentration, while the reverse occurred with increased initial biomass. The optimum pH and temperature for Co^{2+} removal were 7.0 and 30°C, respectively. Co^{2+} uptake was inhibited in the presence of other metals (Pb, Cd, Cu, Ni, Cr, and Zn), however (Pal et al. 2006). Untreated, heat- and alkali-treated Lentinus sajor-caju (white rot fungus) mycelia were used for the recovery of U from aqueous solution by Bayramoğu et al. (2006). He reported that the alkali-treated form had a high biosorption capacity (378 mg/g) compared with 268 mg/g for untreated and 342 mg/g for heat-treated fungal mycelia. Optimum biosorption was observed at pH 4.5 for all the tested fungal preparations and was independent of temperature (5–35°C). Naeem et al. (2006) studied H^+, Cd, Pb, Sr, and Zn adsorption onto *S. cerevisiae*. They modeled the acid/base properties of the fungal cell wall by invoking a nonelectrostatic surface complexation model with four discrete surface organic acid functional group types, with average *p*Ka values of 3.4 ± 0.4, 5.0 ± 0.2, 6.8 ± 0.4, and 8.9 ± 0.6. The affinity of the fungal cells for the metal ions followed the trend: Pb > Zn > Cd > Sr. The authors used the metal adsorption data to determine site-specific stability constants for the important metal fungal surface complexes. Their results showed that *S. cerevisiae* may represent a novel biosorbent for the removal of heavy metal cations from aqueous waste streams. Pokhrel and Viraraghavan (2006) reported potential removal of As from an aqueous solution by nonviable fungal biomass of *A. niger* coated with Fe. *A. niger* biomass coated with

iron oxide showed maximum removal (approximately 95% of As^{5+} and 75% of As^{3+}) at pH 6. No strong relationship was observed between the surface charge of the biomass and As removal.

Biosorption of Zn, Cu, Hg, Cd, or Pb by living or nonliving biomass of *A. macrosporus* from an acid solution, acid solution supplemented with P and K, and an alkaline solution showed maximum uptake of all metals (Cu 96% and Pb 89%) at alkaline pH. With living biomass, metal biosorption was greater and more rapid in P/K-supplemented acid media than in nonsupplemented acid media (Melgar et al. 2007). Zafar et al. (2007) observed in vitro Cr and Cd biosorption capacity among fungi isolated from wastewater-treated soil, which belonged to genera *Aspergillus*, *Penicillium*, *Alternaria*, *Geotrichum*, *Fusarium*, *Rhizopus*, *Monilia*, and *Trichoderma*. Maximum biosorption of Cr and Cd ions was detected at 6 mM initial metal concentration. *Aspergillus* sp.1 accumulated 1.2 mg of Cr and 2.7 mg Cd/g of biomass. Accumulation of these metals by metal-tolerant *Aspergillus* sp.2 isolate was at par with relatively less tolerant *Aspergillus* sp.1 isolate. *Rhizopus* sp. accumulated 4.3 mg of Cr and 2.7 mg Cd/g of biomass. These findings indicate promising capabilities for biosorption of Cd and Cr by *Rhizopus* and *Aspergillus* spp. from aqueous solution. There is little, if any, correlation between metal tolerance and biosorption properties of the test fungi.

Bishnoi et al. (2007) reported that the biosorption efficiency of powdered *T. viride* biomass entrapped in a polymeric matrix of calcium alginate compared with that of cell-free calcium alginate beads. Biosorption of Cr^{6+} was pH-dependent and maximum adsorption (16.1 mg/g) was observed at pH 2.0. The maximum adsorption capacity was observed at a dose of 0.2 mg in 100 ml of Cr^{6+} solution. The experimental results were fitted satisfactorily to both Langmuir and Freundlich isotherm models. The hydroxyl (–OH) and amino (–NH) functional groups were responsible for the biosorption of Cr^{6+} with fungal biomass.

Fungal strain *T. harzianum* was found to be a comparatively better candidate for uranium biosorption than algae. The process was highly pH-dependent. At optimized experimental parameters, the maximum uranium biosorption capacity of *T. harzianum* was 612 mg U g^{-1}, whereas maximum values of uranium biosorption capacity exhibited by algal strains (RD256 and RD257) were 354 and 408 mg U g^{-1}, respectively, and much higher in comparison with commercially available resins (Dowex-SBR-P and IRA-400). Uranium biosorption by algae followed the Langmuir model while fungus exhibited a more complex multilayer phenomenon of biosorption and followed pseudo-second-order kinetics. Mass balance studies revealed that uranium recovery was 99.9% for *T. harzianum*, and 97.1 and 95.3% for RD256 and RD257, respectively, by 0.1 M hydrochloric acid, which regenerated the uranium-free cell biomass facilitating the sorption–desorption cycles for better economic feasibility (Akhtar et al. 2007)

Das and Guha (2007) found biomass of *Termitomyces clypeatus* (TCB) to be the most effective for biosorption of all fungal species tested. Sorption of Cr^{6+} by live TCB depends on pH of the solution, with the optimum pH value being 3.0. The biomass amino, carboxyl, hydroxyl, and phosphate groups chemically interacted with the chromate ion forming a cage-like structure as depicted by scanning electron microscopic

(SEM) and Fourier-transformed infrared spectroscopic (FTIR) results. Desorption and FTIR studies also showed that Cr^{6+} was reduced to trivalent chromium on binding to the cell surface. The maximum Pb^{2+} biosorption capacity of *Aspergillus parasiticus* was found to be 4.02×10^{-4} mol/g at pH 5.0 and 20°C in a batch system. The biosorption equilibrium was reached in 70 min (Akar et al. 2007).

Chen and Wang (2007) used waste biomass of *Saccharomyces* as a biosorbent to react with ten metal ions, and maximum biosorption capacity (q (max)) was determined by the Langmuir isotherm model. They reported that values of q (max) decreased in the following order (in mM/g): Pb^{2+} (0.413) > Ag^{+} (0.385) > Cr^{3+} (0.247) > Cu^{2+} (0.161) > Zn^{2+} (0.148) > Cd^{2+} (0.137) > Co^{2+} (0.128) > Sr^{2+} (0.114) > Ni^{2+} (0.108) > Cs^{+} (0.092). It is suggested that the greater the covalent index value of the metal ion, the greater the potential to form covalent bonds with biological ligands such as sulfhydryl, amino, carboxyl, hydroxyl, etc. on the biomass surface and the higher the metal ion biosorption capacity. Fan et al. (2009) studied the isotherms, kinetics, and thermodynamics of Cd^{2+}, Zn^{+2}, and Pb^{2+} biosorption by *Penicillium simplicissimum* in a batch system. The effects of pH, initial metal ion concentration, biomass dose, contact time, temperature and presence of co-ions on biosorption were studied. The results of the kinetic studies at different temperatures showed that adsorption rate followed pseudo-second-order kinetics. The thermodynamic constants ΔG^{o}, ΔH^{o}, and ΔS^{o} of the adsorption process showed that biosorption of Cd^{2+}, Zn^{+2}, and Pb^{2+} ions on *P. simplicissimum* were endothermic and spontaneous.

The quantity of metals retained through bioaccumulation by fungal strains *Penicillium sp.* A1 and *Fusarium* sp. A19 and of a consortium of the two types of strains (A1+A19) was significantly higher than that through biosorption by these fungi. The highest quantities of Cd, Cu, and Zn accumulated by fungal biomass was obtained in the presence of $Cd^{2+} + Cu^{2+} + Zn^{2+}$ in potato dextrose agar compared with the individual A1 or A19 used in PDB. A1+A19 accumulated greater quantities of Cu and Pb in the presence of $Cd^{2+} + Cu^{2+} + Pb^{2+}$ and greater quantities of Pb in the presence of $Cd^{2+} + Cu^{2+} + Zn^{2+} + Pb^{2+}$. There was no simple relationship between metal biosorption by fungal biomass and fungal metal tolerance. The biomass of A1 + A19 cultivated in PDB absorbed greater quantities of metals than A1 or A19 in the presence of single metals and their combinations (Pan et al. 2009). The results suggest that the applicability of growing fungi tolerant to heavy metals provides a potential biotechnology system for the treatment of wastewaters contaminated with heavy metals (Pan et al. 2009).

Effect of biosorbent dosage, initial solution pH and initial Ni^{2+} concentration on uptake of Ni^{2+} by NaOH-pretreated biomass of *A. niger* from aqueous solution was investigated by Amini et al. (2009). Optimum Ni^{2+} uptake (4.8 mg Ni^{2+}/g biomass, 70.3%) was achieved at pH 6.25, biomass dosage 2.98 g/L, and initial Ni^{2+}concentration 30.0 mg/L Ni^{2+}. Langmuir and Freundlich isotherms described the biosorption fairly well; however, the prediction of Ni^{2+} biosorption using Langmuir and Freundlich isotherms was relatively poor in comparison with response surface methodology (RSM) approaches. Pakshirajan and Swaminathan (2009) studied biosorption of Cu^{2+} and Cd^{2+} by live *Phanerochaete chrysosporium*

immobilized by growing onto polyurethane foam in individual packed bed columns over two successive cycles of sorption–desorption. Initial pH and metal concentrations in their respective solutions were set to optimal levels (4.6 and 35 mg/L in the case of Cu and 5.3 and 11 mg/L for Cd). The breakthrough curves obtained for the two metals during sorption in both cycles exhibited a constant pattern at various bed depths in the columns. The maximum yield of the columns in removing these metals was found to be 57 and 43% for Cu and Cd, respectively. Recovery values of the sorbed Cu and Cd from the respective loaded columns using 0.1 N HCl as eluant exceeded 65 and 75%, respectively, at the end of desorption in both the cycles. In recent years, Tsekova et al. (2010) reported biosorption of Cu^{2+} and Cd^{2+} from aqueous solution by free and immobilized biomass of *A. niger*. Tsekova et al. (2010) study investigated the ability of *A. niger* resting cells entrapped in a polyvinyl alcohol (PVA) network to remove Cu^{2+} and Cd^{2+} from single-ion solutions. The performance of free and immobilized biosorbent was evaluated by equilibrium and kinetic studies. The PVA-immobilized fungal biosorbent removed Cu^{2+} and Cd^{2+} rapidly and efficiently with maximum metal removal capacities of 34.1 and 60.2 mg/g, respectively. These values of metal uptake at equilibrium were higher than the quantity of Cu^{2+} and Cd^{2+} removed by free biomass (17.6 and 69.4 mg/g, respectively). Biosorption equilibrium data were best described by Langmuir isotherm models. The biosorption kinetics followed the pseudo-second-order model and intraparticle diffusion equation. The results obtained suggest that the immobilized biosorbent holds great potential for wastewater treatment applications.

Based on the above literature search, it is concluded that there is sufficient scientific data on the potential exploitation of fungal biomass for heavy metal removal from aqueous solutions. Therefore, further efforts should be focused on the development of specific technologies for metal removal and recovery from fungal biomass systems. More data are needed to assess the factors influencing metal removal in wastewater treatment systems and to build upon these issues.

13.8 Conclusions

Many modes of nonactive metal removal by microbial biomass are documented. Any one or a combination can be functional in immobilizing metallic species on biosorbents. Soil fungi seem to be well adapted to metals and could effectively be used as a metal biosorbent, either in living, dead and/or immobilized states. Metal tolerance appears to be an added advantage when using live cells for metal removal. A number of anionic ligands participate in metal removal: phosphoryl, carbonyl, sulfhydryl, and hydroxyl groups can all be active to various degrees in binding the metal. Due to the accumulated knowledge and due to the significant economic margin for application in metal removal/detoxification processes, new biosorbent materials are currently well poised for commercial exploitation. However, there are no limits to expanding the science of biosorption required to provide a deeper understanding of the phenomenon and to support effective application attempts.

Human populations need technologies to treat water supplies and diminish the environmental dangers posed by certain industrial and agricultural practices. Biosorption can be a solution to providing abundant clean water and treating soils contaminated by heavy metals. Research in the past two decades has provided a better understanding of metal sorption by certain potential biosorbents. Application aspects are being aimed at biosorption process optimization and development of strategies for further processing of biosorbent as a greener and cleaner technology.

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Chapter 14
Rhizosphere and Root Colonization by Bacterial Inoculants and Their Monitoring Methods: A Critical Area in PGPR Research

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Abstract Roots serve a multitude of functions in plants including anchorage, acquisition of nutrients and water, and production of exudates with growth regulatory properties. The root–soil interface, or rhizosphere, is the site of greatest biological and chemical activity within the soil matrix. Plant growth-promoting rhizobacteria (PGPR) are known to influence plant health by controlling plant pathogens or via direct enhancement of plant development in the laboratory and in greenhouse experiments. Unfortunately, however, results in the field have been less consistent. The colonization of roots by inoculated bacteria is an important step in the interaction between beneficial bacteria and the host plant. However, colonization is a complex phenomenon influenced by many biotic and abiotic parameters, some of which are only now apparent. Monitoring fate and metabolic activity of microbial inoculants as well as their impact on rhizosphere and soil microbial communities are needed to guarantee safe and reliable application, independent of whether they are genetically modified or not. The first and most crucial prerequisite for effective use of PGPRs is that strain identity and activity are continuously confirmed. A combination of both classical and molecular techniques must be perfected for more effective monitoring of inoculants strain (both genetically modified and unmodified) after release into the soil. Recent developments in techniques for studying rhizobacterial communities and detection and tracking systems of inoculated bacteria are important in future application and assessment of effectiveness and consistent performance of microbial inoculants in crop production and protection.

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_14,

14.1 Introduction

The importance of rhizosphere microbial populations for maintenance of plant root health, nutrient uptake, and tolerance of environmental stress is well recognized (Bowen and Rovira 1999). These beneficial microorganisms can be a significant component of management practices to achieve attainable crop yields, defined as yields limited only by the natural physical environment of the crop and its innate genetic potential (Cook 2002). The prospect of manipulating crop rhizosphere microbial populations by inoculation of beneficial bacteria to increase plant growth has shown considerable promise in laboratory and greenhouse studies; however, responses have been variable in the field (Bowen and Rovira 1999). The potential environmental benefits of this approach, including healthy sustainable management practices with a reduction in use of agricultural chemicals are driving this technology. Recent progress in our understanding of the biological interactions that occur in the rhizosphere and of the practical requirements for microbial inoculant formulation and delivery systems will increase this technology's consistency in the field and facilitate its commercial development.

Plant growth-promoting rhizobacteria (PGPR) were first defined by Kloepper and Schroth (1978) to describe soil bacteria that colonize the roots of plants following inoculation onto seeds, and that enhance plant growth. Those components which comprise the colonization process include the ability to (1) survive inoculation onto seed; (2) multiply in the spermosphere (region surrounding the seed) in response to seed exudates; (3) attach to the root surface; and (4) colonize the developing root system (Kloepper 1994). The ineffectiveness of PGPR in the field has often been attributed to their inability to colonize plant roots (Benizri et al. 2001; Bolemberg and Lutenberg 2001). A variety of bacterial traits and specific genes contribute to root colonization, but only a few have been identified (Benizri et al. 2001; Lugtenberg et al. 2001) and include motility, chemotaxis to seed and root exudates, production of pili or fimbriae, production of specific cell surface components, ability to use specific components of root exudates, protein secretion, and recently biofilm-forming ability of the microbes and quorum sensing (Lugtenberg et al. 2001; Sharma et al. 2003). The generation of mutants altered in expression of these traits is aiding our understanding of the precise role each plays in the colonization process (Lugtenberg et al. 2001; Persello-Cartieaux et al. 2003). Progress in the identification of new, previously uncharacterized genes is being made using nonbiased screening strategies that rely on gene fusion technologies. These strategies employ reporter transposons (Roberts et al. 1999) and in vitro expression technology (IVET) (Rainey 1999) to detect genes expressed during colonization.

Using molecular markers such as green fluorescent protein (GFP) or fluorescent antibodies, it is possible to monitor the location of individual rhizobacteria on the root using confocal laser scanning microscopy (Bloemberg et al. 2000; Bolemberg and Lutenberg 2001; Sorensen et al. 2001). This approach has also been combined with an rRNA-targeting probe to monitor the metabolic activity of specific rhizobacterial strains, and showed that bacteria located at the root tip were most active (Lubeck et al. 2000; Sorensen et al. 2001).

An important aspect of colonization is the ability to compete with indigenous microorganisms present in the soil and rhizosphere of the developing plant. Our understanding of the factors involved in these interactions has been hindered by our inability to culture and characterize diverse members of the rhizosphere community and to determine how that community varies with plant species, plant age, location on the root, and soil properties. Phenotypic and genotypic approaches are now available to characterize rhizobacterial community structure. Phenotypic methods that rely on the ability to culture microorganisms include standard plating methods on selective media, community level physiological profiles (CLPP) using the BIOLOG system (Garland 1996), phospholipid fatty acid (PLFA) (Tunlid and White 1992), and fatty acid methyl ester (FAME) profiling (Germida et al. 1998). Culture-independent molecular techniques are based on direct extraction of DNA from soil and 16S-rRNA gene sequence analysis, bacterial artificial chromosome or expression cloning systems (Rondon et al. 1999). These are providing new insights into the diversity of rhizosphere microbial communities, the heterogeneity of the root environment, and the importance of environmental and biological factors in determining community structure (Baudoin et al. 2002; Berg et al. 2002; Smalla et al. 2001). These approaches can also be used to determine the impact of inoculation of PGPR on the rhizosphere community (Ciccillo et al. 2002; Steddom et al. 2002). Various microbial inoculants are used to treat plant seeds and seedling roots to promote plant growth and protect plant health. Numerous factors, both biotic and abiotic, are known which influence the performance of inoculated bacteria under field conditions. These factors may influence inoculants survival, colonization, and establishment in the rhizosphere; however, in many cases no assessment has been made to detect the presence and colonization of inoculated bacteria in the rhizosphere. This has primarily been due to the absence of selection criteria in the inoculants strain to distinguish it from indigenous bacteria. Recent advances in molecular techniques have given hope to developing inoculants with specific markers to be included for detection and colonization in the rhizosphere and to assess their performance. The present chapter aims to provide an overview of rhizosphere colonization by rhizobacteria and methods used to detect, identify, and monitor colonization by inoculated bacteria in the root zone to ensure more effective and consistent performance of inoculants under field conditions.

14.2 The Rhizosphere and Rhizospheric Effect

The term “rhizosphere” was first used by Hiltner (1904) to describe the zone of soil under the influence of plant roots. The rhizosphere is the area of increased microbial diversity and activity. From about 0 to 2 mm from the root surface the soil is significantly influenced by living roots. The rhizosphere can thus be described as the longitudinal and radial gradients occurring with expanding root growth, nutrient and water uptake, exudation, and subsequent microbial growth (Uren 2000). The rhizosphere is important in terms of root growth, exudate production, and community development of both macro- and microbiota. Stimulation of microbial proliferation

around the root due to the release of various organic compounds by the roots is known as the rhizospheric effect. The ability to secrete a vast array of compounds into the rhizosphere is one of the most remarkable metabolic features of plant roots, with nearly 49% of all photosynthetically fixed carbon being transferred to the rhizosphere through root exudates (Kennedy 1999).

14.2.1 Rhizosphere Colonization

Root exudates released into the soil environment from plants have been traditionally grouped into low- and high-molecular weight compounds. High-molecular weight compounds include polysaccharides, mucilage, and proteins. Plant mucilages are released from the root cap, the primary cell wall between epidermal and sloughed root cap, and epidermal cells (including root hairs). Lysates are released from roots during autolysis. Rhizospheric microorganisms also release microbial mucilages. Collectively, plant and microbial mucilages, microbial cells and their products together with associated organic and mineral matter are referred to as mucigel (Walker et al. 2003). Low-molecular organic compounds released by plant roots include ethylene, sugars, amino acids, vitamins, polysaccharides, and enzymes (Table 14.1). The fact the nutritional resources influence population structure and play a role in niche colonization and competition. The microbial population in and around roots includes bacteria, fungi, yeasts, and protozoa. Bacterial populations in

Table 14.1 Compounds and enzymes identified in plant root exudates

Class of compounds	Type of compounds
Amino acids	Alanine, α-aminoadipic acid, γ-aminobutyric acid, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, histidine, homoserine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, therionine, tryptophan, tyrosine, valine
Organic acids	Acetic, aconitic, aldonic, butyric, citric, erythronic, formic, fumaric, glutaric, glycolic, lactic, malic, malonic, oxalic, piscidic, propionic, pyruvic, succinic, tartaric, tertronic, valeric acid
Sugars	Arabinose, deoxyribose, fructose, galactose, glucose, maltose, oligosaccharides, raffinose, rhamnose, ribose, sucrose, xylose
Vitamins	*p*-Aminobenzoic acid, biotin, choline, *n*-methionylnicotinic acid, niacin, panthothenate, pyridoxine, riboflavin, thiamine
Fatty acids and sterols	Palmitic, stearic, oleic, linoleic, and linoleic acids; cholesterol, campestrol, stigmasterol, sitosterol
Nucleotides	Adenine, guanine, uridine, cytidine
Enzymes	Amylase, invertase, phosphatase, polygalactouranase, proteases
Miscellaneous	HCO_3^-, OH^-, H^+, CO_2, H_2; auxins, flavonones, glycosides, saponin, scopolotin

Sundin (1990); Bolton et al. (1993); Dakora and Philipps (2002); Bais et al. (2006); Ahmad et al. (2008)

the rhizosphere are predominantly Gram-negative short rods including species of *Pseudomonas*, *Flavobacterium*, and *Alcaligenes*, etc. Some are free-living while others form symbiotic associations with plants. The interaction between microorganisms and roots may be beneficial, harmful, or neutral for the plant and sometimes the effect of microorganisms may vary as consequence of soil conditions (Alexander 1985; Lynch 1990; Ahmad 2006).

Root colonizers may be pathogenic, symbiotic, and plant growth-promoting microorganisms. Based on these activities, the plant-beneficial microorganisms can be classified as biofertilizers, phytostimulators, rhizoremediators, and biopesticides. Despite their importance to plant growth, the molecular basis of colonization in these plant–microbe interactions is not completely understood. This is a key reason for the limited success of PGPR in field conditions.

Plant root colonization by a bacterium can be considered as an enrichment of the best adapted microorganisms to a particular ecological niche. The colonization of the volume of soil under the influence of the root is known as rhizosphere colonization (Kloepper et al. 1991; Kloepper 1994). Rhizosphere colonization is important not only as the first step in pathogenesis of soil-borne microorganisms but is also crucial in the application of microorganisms for beneficial purposes. A variety of bacterial traits and specific genes contribute to colonization but few have been identified (Lugtenberg et al. 2001; Benizri et al. 2001). PGPR generally improves plant growth by colonizing the root system and pre-empting the establishment of, or suppressing deleterious rhizosphere microorganisms (Schroth and Hancock 1982). PGPR must be able to compete with indigenous microorganisms and efficiently colonize the rhizosphere of the plant to be protected; such colonization is widely believed to be essential for biocontrol (Weller 1983; Parke 1991). Thus, a biocontrol agent should be able to proliferate and ultimately colonize the surface of the plant root (Benizri et al. 2001; Bolemberg and Lutenberg 2001).

Colonization of roots by inoculated bacteria is an important step in the interaction between beneficial bacteria and the host plant. Seed colonization is the first step in root colonization. Microorganisms established on the germinating seed can multiply and colonize the root as it emerges and grows through soil. Thus colonization of the imbibing seed may predispose future colonization of the root (Sylvia et al. 1999).

The competitive exclusion of deleterious rhizosphere organisms is directly linked to an ability to successfully colonize a root surface. In effect, all disease-suppressive mechanisms demonstrated by florescent pseudomonads are essentially of no real value unless these bacteria can successfully establish themselves in the root environment (Kloepper et al. 1980; De Weger et al. 1987).

14.2.2 Competition for Root Niches and Bacterial Determinants Directly Involves Root Colonization

The root surface and surrounding rhizosphere are significant carbon sinks (Rovira 1965). Photosynthate allocation to this zone can be as high as 40% (Degenhardt

et al. 2003). Thus, along root surfaces there are various suitable nutrient-rich locations attracting a diversity of microorganisms including phytopathogens. Competition for nutrients and niches is a fundamental mechanism by which PGPR protects plants from phytopathogens (Duffy 2001).

The quantity and composition of chemoattractants and antimicrobials exuded by plant roots are under genetic and environmental control (Bais et al. 2004b). This implies that PGPR competence strongly depends either on their abilities to take advantage of a specific environment or on their abilities to adapt to changing conditions. PGPR may be uniquely equipped to sense chemo attractants, e.g., rice exudates induce stronger chemotactic responses of endophytic bacteria as compared to non-PGPR present in the rice rhizosphere (Bacilio-Jime´nez et al. 2003). Bacterial lipopolysaccharides (LPS), in particular the O-antigen chain, also contribute to root colonization (Dekkers et al. 1998a). However, the importance of LPS in such colonization might be strain-dependent since the O-antigenic side chain of *Pseudomonas fluorescens* WCS374 does not contribute to potato root adhesion (De Weger et al. 1989), whereas the O-antigen chain of *P. fluorescens* PCL1205 is involved in tomato root colonization (Dekkers et al. 1998a). Furthermore, the O-antigenic aspect of LPS does not contribute to rhizoplane colonization of tomato by the plant-beneficial endophytic bacterium *P. fluorescens* WCS417r; however, this bacterial determinant was involved in endophytic colonization of roots (Duijff et al. 1997). It has also been recently demonstrated that the high-bacterial growth rate and ability to synthesize vitamin B1 and exude NADH dehydrogenases contribute to plant colonization by PGPR (Dekkers et al. 1998a; Simons et al. 1996). Another determinant of root colonization ability by bacteria is type IV pili, better known for its involvement in the adhesion of animal and human pathogenic bacteria to eukaryotic cells (Strom and Lory 1993). The type IV pili also play a role in plant colonization by endophytic bacteria such as *Azoarcus* sp. (Steenhoudt and Vanderleyden 2000; Compant et al. 2005). Bacterial traits required for effective root colonization are subject to phase variation, a regulatory process for DNA rearrangements orchestrated by site-specific recombinase (Dekkers et al. 1998b; Sa´nchez-Contreras et al. 2002; Van der Broek et al. 2003). In certain PGPR, efficient root colonization is linked to their ability to secrete a site-specific recombinase (Dekkers et al. 1998b; Dennis et al. 2010). Transfer of the site-specific recombinase gene from a rhizosphere-competent *P. fluorescens* into a rhizosphere-incompetent *Pseudomonas* strain enhanced its ability to colonize root tips (Dekkers et al. 2000; Compant et al. 2005; Martínez-Granero et al. 2005; Mavrodi et al. 2006).

14.2.3 Biofilms in the Rhizosphere

Bacteria adhere to environmental surfaces in multicellular assemblies described as biofilms. Plant-associated bacteria interact with host tissue surfaces and form biofilm-type structure to extensive mature biofilm. The surface properties of plant tissue,

nutrient and water availability, and the proactivities of the colonizing bacteria strongly influence biofilm structure. Biofilms hold tremendous practical significance in agricultural, industrial, and medical settings, exhibiting both beneficial and detrimental activities (Webb et al. 2003; Parsek and Fuqua 2004; Ramey et al. 2004).

Root-associated pseudomonads have been studied extensively and many promote the growth of host plants or are used as biocontrol agents (Lugtenberg et al. 2001). Plant growth-promoting pseudomonads have been reported to discontinuously colonize the root surface, developing as small biofilms along epidermal fissures (Bloemberg et al. 2000). However, pathogenic pseudomonads formed dense, confluent biofilms (Walker et al 2004; Bais et al. 2004a). It has been suggested that pseudomonad root biofilms can range from relatively small multicellular clusters to extensive biofilm networks (Ramey et al. 2004). The root colonization ability of *Azospirillum brasience* and other related species with cereals such as wheat and maize is widely known. It has been found that plant growth promotion by this free-living diazotrophic bacteria is associated with colonization on root elongation zones and root hairs followed by the formation of biofilm (Assmus et al. 1995). The ability of rhizobia to form root nodules is also associated with biofilm formation in curled root hairs. *Agrobacterium tumefaciens* and rhizobia form dense biofilms on root surfaces, extensively coating the epidermis and root hairs as well as abiotic surfaces. *Bacillus cereus*, a Gram-positive bacteria, develops dense surface-associated populations, and one recent study has linked biocontrol with the ability of this species to form biofilms (Bais et al. 2004a). Several functions known to influence biocontrol activity are also likely to play a role in biofilm formation (Dunn et al. 2003).

A number of microbial cell structures such as flagella or type IV pili, LPS, and outer membrane proteins including adhesins are important in colonization and biofilm formation (vande Broek and Venderleyden 1995; Tans-Kersten et al. 2001; Hinsa et al. 2003). Similarly, bacterial products such as exopolysaccharides are well-associated with biofilm development in many bacteria including *Pseudomonas aeruginosa* and *A. tumefaciens* (Ramey et al. 2004).

Biofilm-forming ability of various pathogenic bacteria are now widely known and described. Ramey et al. (2004) have described biofilm formation in vascular pathogens such as *Xylella fasttidiosa*, *Xanthomonas campestris* pv *campestris*, *Pantoea stewartii* sub sp. *stewartii*, *Ralstonea solanacearum*, and *Clavibacter michiganensis*. Many bacterial biofilm formation processes are linked with a cell–cell communication mechanism termed quorum sensing. Many other bacteria including pathogenic, symbiotic, and free-living have been increasingly demonstrated to form biofilms on biotic and abiotic surfaces. Various factors including surface chemistry, and nutrient availability and intrinsic ability of bacteria determine the nature and types of biofilm formed. An important issue for future research efforts is determination of the extent of contribution of biofilms in plant growth promotion by different rhizospheric bacteria, especially root-associated free-living bacteria (Timmusk et al. 2005; Rudrappa et al. 2008).

Recent reports on quorum sensing and its modification due to the presence of plant root exudates/metabolites have further compounded the complexity of microbe–plant root interaction mechanisms. Inter- and intra-species signal molecules

(*N*-acyl homoserine lactones for Gram-negative bacteria and protein/polypeptides for Gram-positive bacteria) are synthesized as bacterial populations reach high densities and play a part in regulating sets of genes involved in the production of exoenzymes such as pectin lyase, pectate lyase, polygalactouranase, cellulase, and protease (Fray et al. 1999) or antibiotics (Pierson et al. 1998). Thus, root colonization by rhizobacteria is usually found to be correlated with high inoculum density (Berger et al. 1995; Pillay and Nowak 1997; Wei and Zhang 2006; Scott et al. 2006; Williams 2007; Klein et al. 2009).

14.2.4 Factors Affecting Root Colonization and Efficacy of Rhizobacteria

Bacterial root colonization is primarily influenced by the presence of specific bacterial traits required for attachment and subsequent establishment; however, other abiotic and biotic factors play an important role in colonization. When an organism colonizes a root, the process must be confirmed with an array of external parameters including water content, temperature, pH, soil types (texture, organic matter, microaggregate stability, presence of key nutrients such as N, P, K, and Fe), composition of root exudates, and presence of other microorganisms. Plant species is another major determinant of overall microbial diversity (Grayston et al. 1998; Dakora and Philipps 2002). The colonization of a fluorescent *Pseudomonas* strain in the potato rhizosphere was reported to be tenfold greater in a sandy loam soil than in clay loam soil (Benizri et al. 2001).

Root colonization of bacteria is negatively affected by predation (protozoa) and parasitism (bacteriophages). Inoculated bacteria must compete with natural inhabitants of the soil for nutrients. The biosynthesis of antagonistic compounds by rhizobacteria such as antibiotics could be affected by nutrient competition. Antibiotic secretion also plays an important role in the establishment of bacteria in the rhizosphere (De Weger et al. 1995; Greer-Phillips et al. 2004; De Weert and Bloemberg 2006).

In vitro activities exhibited by various PGPR for biocontrol may not provide the identical results under field conditions. The failure of PGPR to produce the desired effects after seed/seedling inoculation is frequently associated with their inability to colonize plant roots. The process of root colonization is complex; several traits associated with survivability, tolerance, competition with indigenous rhizospheric microorganisms, and expression of root colonizing traits are important (Somers and Vanderleyden 2004). In many countries, harsh climatic conditions, population pressures, land constraints, and decline of traditional soil management practices have often reduced soil fertility. Such extreme effects will certainly alter soil's chemical, physical, and biological properties and therefore affect microbial colonization.

Biocontrol agents may be affected by indigenous soil microbial communities and they may also influence the community into which they are introduced. Enhancement of introduced PGPR populations leading to enhanced suppression of

pathogens can be augmented by manipulation of several field cultural practices (Kloepper et al. 1999). This may include application of organic matter (Siddiqui 2004; Siddiqui 2006).

A single biocontrol agent is not active against all the pathogens that attack the host plant; a single biocontrol agent is effective against a single pathogen under laboratory conditions. This may be the reason for the inconsistent performance of biocontrol agents introduced into the field. Naturally occurring biocontrol results from mixtures of agents, rather than from high populations of a single organism. Greater suppression and enhanced consistency against multiple cucumber pathogens were observed using strain mixtures of PGPR (Raupach and Kloepper 1998). Incompatibility of the co-inoculants may sometimes arise and thus inhibit each other as well as the target pathogens (Leeman et al. 1996). This is therefore an important prerequisite for successful development of strain mixtures. Even more important is that the inoculant strains may fail to survive and not colonize the root. Patterns of survival and effectiveness with growth phases of plants have not been clearly studied; nor have efforts to distinguish inoculated PGPR from indigenous microbial populations. Thus, various methods are in use to monitor inoculant strains, both genetically modified and nonmodified. Some techniques are briefly described below.

14.3 Monitoring of Microbial Inoculants (Biocontrol Agents/PGPR)

Substantial range of monitoring methods has been developed for the detection and quantification of microorganisms for various purposes (Morris et al. 2002). Monitoring methods can be divided into three groups: microbiological, direct methods, and molecular methods. Here, a brief descriptions of the common methods used to monitor biocontrol agents are described (Table 14.2).

14.3.1 Microbiological Monitoring Methods

These methods are culture-based classical methods and are commonly used to study and monitor soil microbes including those inoculated into the soil system for their survival and colonization on root surfaces as well as in bulk soil. The basic requirement for such methods is the availability of selective media for target organisms to differentiate from native microbes. It is at times difficult to differentiate inoculated organisms from native populations based on morphological characteristics (Lima et al. 2003). Many authors have used the spontaneous mutant of the parent strain resistant to antibiotics such as nalidixic acid and rifampicin in order to differentiate with indigenous bacterial population (Nautiyal 2000; Lindow and Suslow 2003; Ahmad et al. 2006). However, resistance to antibiotics among indigenous populations which can grow on selective media should be first checked before

Table 14.2 Monitoring methods of bacterial and fungal biocontrol agents

Species	Strain	Detection	Quantification	References
Bacteria				
P. agglomerans	C9-1	Ab-R	CFU count	Nuclo et al. 1998
P. agglomerans	EPS125	Ab-R	CFU count	Bonnaterra et al. 2005
P. agglomerans	Eh24	Ab-R	CFU count	Ozaktan and Bora 2004
P. corrugata	2140	Ab-R, lacZY gene rep-PCR	CFU count	Choi et al. 2003
P. fluorescens	A506	Ab-R, gfp gene	CFU count, FCM, microscopy, fluor	Nuclo et al. 1998; Lowder et al. 2000; Lindow and Suslow 2003
P. fluorescens	EPS62e	Ab-R	CFU count	Cabrefiga 2004
P. fluorescens	EPS288	Ab-R	CFU count	Montesinos and Bonaterra 1996
P. fluorescens	SBW25	gfp, lux genes	CFU count, FCM, Lum.	Unge et al. 1999
P. fluorescens	phlD	Ab-R, rep-PCR	CFU count, Hybr., MPN-PCR	Landa et al. 2002
P. fluorescens	29A	Ab-R, RAPD gfpmut2 gene, SCAR	CFU count QC-PCR	Chapon et al. 2002, 2003
P. fluorescens	CHAO	phlA gene	CFU count QC-PCR	Rezzonico et al. 2003
Fungi				
Hirsutella rhossiliensis	OWVT-1	ITS sequence	Real-time PCR	Zhang et al. 2006
Paecilomyces lilacinus	RESP11, 251	ITS sequence	CFU count, real-time PCR	Atkins et al. 2005
Plectosphaerella cuccumerina	380408	ITS sequence	CFU count, real-time PCR	Atkins et al. 2003
Trichoderma hamatum	382	RAPD, SCAR	CFU count-PCR	Abbassi et al. 1999
Trichoderma harzianum	2413	SCAR	Real-time PCR	Rubio et al. 2005
Verticillium chamydosporium	10	β-tubulin gene	CFU count, C-PCR	Mauchline et al. 2002

application. Once a suitable method is developed for the detection of a target organism, a quantitative method based on CFU count and/or most probable number (MPN) should be applied (Russek and Colwell 1983; Rothballer et al. 2003).

However, this technique requires knowledge about traits involved in the colonization process and then to isolate mutants with these traits. For example, studies have focused on motility (Lutenberg et al. 1996), the necessity for biosynthesis of cell surface molecules (Matthysse and McMahan 1998; Lutenberg and Dekkers 1999), O antigens of LPS present in outer membranes, prototrophy for amino acids and vitamin B_1 (Lutenberg et al. 1996; Simons et al. 1997), and growth on seed and root exudates such as carbohydrates and organic acids (Lutenberg and Dekkers 1999; Roberts et al. 1999). To follow the fate of inoculant strains in the rhizosphere of crop plants and nontarget plants, cultivation-dependent methods are most frequently applied. Most suitable for tracking inoculants by selective plating is the use of rifampicin-resistant mutants (i.e., involving mutation of the ribosomal binding site) of the PGPR strains (Lin et al. 2000; Lottmann et al. 2000), as the background level of indigenous soil bacteria with resistance to rifampicin is low. In the past selective plating had been used primarily for strain confirmation; however, nowadays it is understood that these tools, although rapid and inexpensive, are not sufficiently reliable for inoculant strain confirmation since spontaneous mutants (e.g., antibiotic resistance) can readily occur. Although these classical approaches have serious limitations they are still viable due to their simplicity and reproducible nature in many situations, and may provide viable cell counts (Nautiyal 2000).

14.3.2 Direct Monitoring Methods

Direct monitoring methods are based on the detection of a specific phenotypic characteristic of the biological agent, for example the emission of flourescence, to achieve its identification. Bioluminescence is a phenotypic characteristic that can be used to mark biological control/PGPR agents. This technique is based on the introduction of an exogenous reporter gene which encodes for enzymes or proteins responsible for bioluminescence. The most frequently described reporter genes are the *lux* gene from the bacterium *Vibrio fischeri* and *gfp* gene from the jellyfish *Aequorea victoria*. The quantification in direct monitoring is achieved by optical detection methods such as flourescence microscopy (epiflourescence microscopy), spectrofluorometry, or flow cytometry. Many authors using direct monitoring methods for biological control agents in environmental samples make use of gfp markers with flow cytometry (Lowder et al. 2000) and the gfp/lux dual marker with flow cytometry and spectrofluorometry to monitor *P. flourescence* (Unge et al. 1999).

Emphasis has been placed on the detection and enumeration of PGPR released in field inoculations as an essential requirement for the assessment of their survival in field conditions. Fluorescent-antibody and selective plating techniques have served as the conventional strategies for detection and isolation of bacteria in environmental samples (Herbert 1990).

Direct fluorescent antibody (DFA or dFA) (also known as direct immunoflourescence) is a laboratory test that uses antibodies tagged with fluorescent dye that can detect the presence of microorganisms. This method offers straight-forward detection of antigens using fluorescently labeled antigen-specific antibodies. Because detection of the antigen in a substrate of sample (cellular smear, fluid or patient-inoculated culture medium) is the goal, DFA is seldom quantitative.

Immunological techniques are useful for both quantification and in situ visualization of bacteria (Van Vurude and Van-DerWolf 1995; Mahaffee et al. 1997). They are based on specific antibodies directed against bacterial antigens. Compared with the traditional enzyme-linked immunosorbent assay (ELISA) procedure (Tsuchiya et al. 1995), the immunoflourescence colony (IFC) staining approach is more informative since it combines quantification (enumeration of colonies marked with antibodies conjugated with fluorescein isothiocyanate) with visualization in planta. Immunomagnetic attraction (specific antibodies linked to iron oxide particles) is also used for quantification (enumeration of bacteria captured with a supermagnet) (Paulitz 2000). Flourescence-labeled antibodies have been used with success for detection of root-colonizing *Pseudomonas* strains by immunoflourescence microscopy (Kragelund and Nybroe 1996; Troxler et al. 1997).

Monospecific polyclonal antisera raised against *Rhizobium leguminosarum* bv. *trifolii* R39, a bacterium which was isolated originally from redclover nodules, were used to study the colonization of roots of leguminous and nonleguminous plants (*Pisum sativum*, *Lupinus albus*, *Triticum aestivum*, and *Zea mays*) after inoculation.

14.3.3 Molecular Monitoring Methods

Recent developments in molecular detection techniques have greatly increased the ability to track microorganisms and engineered genetic markers in natural environments (Pickup 1991). Molecular biology techniques that allow the detection of microorganisms in soil include the use of DNA probes (Holben et al. 1988), polymerase chain reaction (Steffan and Atlas 1988; Ruppel et al. 2006), use of selective markers such as antibiotic resistance genes, and the use of chromogenic markers such as β-galactosidase (Drahos et al. 1986) and β-glucuronidase (Jefferson 1989). None of the techniques mentioned above provides in situ detection in soil, however. DNA hybridization requires extraction of cells and removal of humic material prior to DNA extraction. For monitoring of organisms after introduction into soil, a selective marker that does not interfere with the ability of the strain to survive and, in the case of microorganisms that interacts with plants, to promote plant growth, is needed.

A general molecular approach to characterize and detect specific microorganism based on direct DNA isolation and subsequent molecular characterization is elaborated in the form of flow chart (Fig. 14.1).

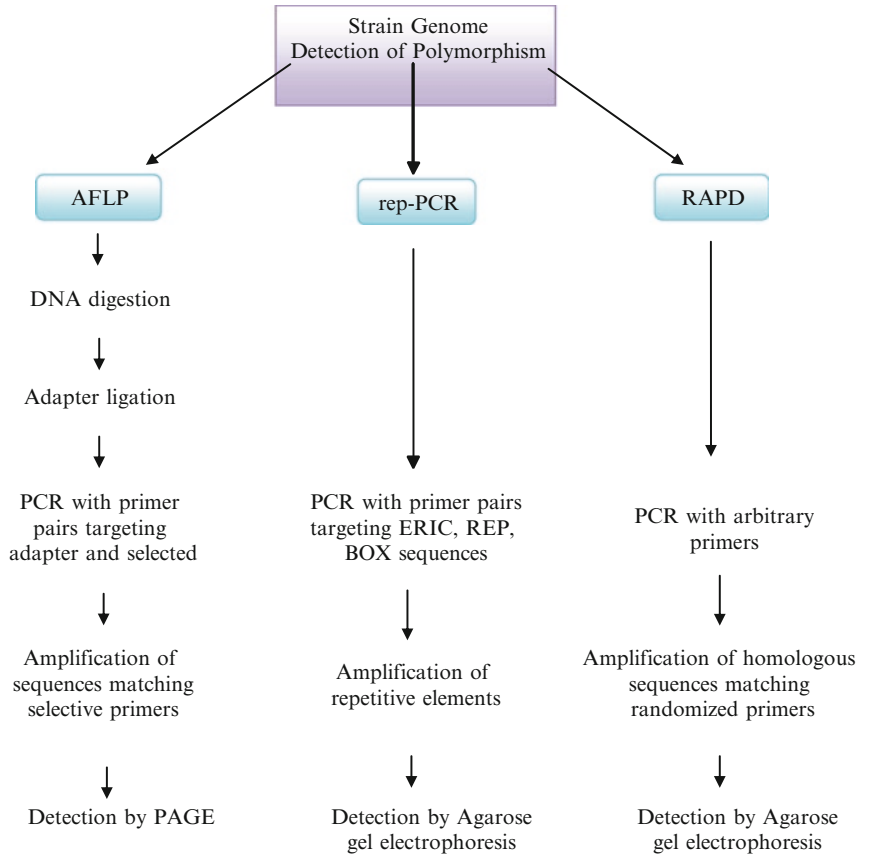

Fig. 14.1 Detection of polymorphism in genome

Many workers have used genomic molecular markers to track the biocontrol strain (Raaijmakers and Weller 2001; Garbeva et al. 2004; Broggini et al. 2005). This technique has drawbacks, as the native strain may also have similar molecular markers. To overcome this problem amplified fragment length polymorphism (AFLP), the amplification of repetitive sequence-based PCR (rep PCR), and random amplified polymorphic DNA (RAPD) are recommended. However, these techniques have been used primarily for eukaryotic organisms (De Curtis et al. 2004; Buhariwalla et al. 2005). AFLP, rep PCR, and RAPD have been used for fingerprinting microorganisms. However, when used for the detection of biological control agents they have a significant drawback; in spite of being specific for characterization of a microorganism, they require the isolation of the target strain prior to its detection. An improvement has been made to the above technique by developing sequence characterized amplified regions (SCARs). SCAR markers are obtained by the selection of a unique amplified fragment which differentiates the target strain from others (Chapon et al. 2003).

Several techniques based on PCR have been developed to achieve quantification as well as detection of target DNA. The first quantification method was PCR-based dilution end point (Q-PCR) (Cross 1995). An improved method has been used for monitoring *P. flourescence* CHA0 (Rezzonico et al. 2003). Improvement in this direction continued and real-time PCR (RT-PCR) has been developed, which is used for monitoring several biocontrol agents, particularly fungi (Schena et al. 2004; Rubio et al. 2005).

Molecular techniques such as rRNA probes, coupled with PCR, are used to quantify and detect inoculated bacteria in plants, labeled probes with fluorochrome (Laguerre et al. 1994; Di Cello et al. 1997; Rothballer et al. 2003; Sørensen et al. 2009; Ruppel et al. 2006), 16S rDNA probe obtained by dot plot hybridization (Amann et al. 1995), enzyme activities quantified by colorimetry *lac*Z (β galactosidases, blue colonies) (Bowen and Rovira 1999), and *gus* A (β glucuronidase, indigo) (Wilson et al. 1994; Wilson et al. 1995).

14.3.4 Use of Reporter Genes

In molecular biology, a reporter gene is a gene that researchers attach to a gene of interest in cell culture, animals, or plants. Certain genes are chosen as reporters because the characteristics they confer on organisms expressing them are readily identified and measured, or because they are selectable markers. Reporter genes are generally used to determine whether the gene of interest has been taken up by or expressed in the cell or organism population. To introduce a reporter gene into an organism, scientists place the reporter gene and the gene of interest in the same DNA construct to be inserted. For bacteria or eukaryotic cells in culture, this is usually in the form of a circular DNA molecule called a plasmid. It is important to use a reporter gene that is not natively expressed in the cell or organism under study, since the expression of the reporter is being used as a marker for successful uptake of the gene of interest (Sørensen and Nybroe 2006).

Commonly used reporter genes that induce visually identifiable characteristics usually involve fluorescent and luminescent proteins; examples include the gene that encodes jellyfish GFP, which causes cells that express it to glow green under blue light, the enzyme luciferase, which catalyzes a reaction with luciferin to produce light, and the red fluorescent protein (RFP) from the gene dsRed. Another common reporter in bacteria is the GUS (UidA) gene, which encodes the protein beta-glucuronidase. This enzyme causes bacteria expressing the gene to appear blue when grown on a medium that contains the substrate analog X-gal (an inducer molecule such as IPTG is also needed under the native promoter). An example of a selectable-marker reporter in bacteria is the chloramphenicol acetyltransferase (CAT) gene, which confers resistance to the antibiotic chloramphenicol (Sørensen and Nybroe 2006; Rochat et al. 2010).

14.3.5 Green Fluorescent Protein

The first application of GFP isolated from the jellyfish *A. victoria* as a reporter (Chalfie et al. 1994) has become a hallmark in modern biology and is used throughout a range of biology and biotechnology research areas including microbiology and cell biology. Advantages in the use of GFP in comparison with other reporters or dyes is that GFP is present within the cell as a product of gene expression and that visualization does not require any fixation or preparation protocols, which are time-consuming and might result in artifacts or influence cellular properties. Furthermore, it does not require substrates or additional energy such as often is the case in bioluminescence. In addition, GFP-labeled cells can be used for flow cytometry analysis and quantitative analysis by PCR (Utermark and Karlovsky 2006). Disadvantages of GFP are that its structure and flourescence are dependent on pH and presence of oxygen (Heim et al. 1994). However, studies on *Rhizobium* tagged with a GFP derivative showed that GFP was well visualized in bacteroids present in root nodules, an oxygen-limiting environment (Gage et al. 1996; Stuurman et al. 2000). A point of consideration before applying GFP is the autoflourescence background or noise from the environment in which the bacteria are to be analyzed. For example, sand and other soil particles as well as certain plant structures or organelles such as chloroplasts can severely hamper GFP visualization. Such problems might be resolved by using other autofluorescent proteins (AFPs) with different excitation and emission wavelength spectra (Bloemberg 2007). Modifications of GFP (often by gene shuffling experiments) have resulted in the isolation of mutants that have shifted emission and excitation wavelengths, which offer the opportunity of using multiple AFPs in one system in order to differentiate between different cells or to visualize different processes within one cell. Important GFP derivatives include enhanced GFP (EGFP), enhanced cyan fluorescent protein (ECFP), and enhanced yellow fluorescent protein (YFP) (Yang et al. 1998; Tsien 1998; Matus 1999; Ellenberg et al. 1999). Blue fluorescent protein (BFP) has also been developed but is less used due to its low brightness. Andersen et al. (1999) have developed a set of GFP derivatives with reduced half-lives by the addition of short amino acid tags to the C terminus, recognized by specific proteases widely present in bacterial cells, which usually break down partially produced proteins. Although efforts have been directed toward isolation of a red fluorescent derivative of GFP, this was never achieved and was bypassed by the discovery and application of RFP or DsRed isolated from the coral *Discosoma striata* (Matz et al. 1999). Since the rfp sequence is not homologous to gfp the use of both genes in one cell will not result in unwanted recombinations. Efficient use of DsRed is hampered by its slow maturation due to its tetramerization, which is required for its fluorescent properties, and its toxic properties when overproduced. Recently, several improved DsRed derivatives have been constructed to overcome these problems. One of these new derivatives, DsRed.T3_S4T, which matures faster (Sorensen et al. 2003), was successfully applied in *Pseudomonas* spp. for rhizosphere studies for being brighter and without causing loss of competitive colonization ability (Dandie et al. 2005). A report by Shaner

et al. (2004) on the construction of improved monomeric red, orange, and YFPs derived from DsRed, which mature more efficiently, are more tolerant to N-terminal fusions and have improved photostability. These forms have not been reported for studies of PGPR.

Over the past few years, the GFP has become a convenient and effective tool for studying microorganisms in complex biological systems. Marker systems based on reporter genes have been widely used to study dynamics and distribution of *gfp*-labeled bacteria in the rhizosphere. GFP-based biosensors allow for detection at the single cell level. The GFP system has numerous advantages over existing marker systems and is especially useful in visualizing spatial distribution and correlation in situ with existing technologies such as confocal laser microscopy or epiflourescence microscopy (Tombolini et al. 1999; Errampalli et al. 1999; Normander et al. 1999; Pallai 2005). The distribution of flourescence levels in populations of cells can be determined using flourescence activated cell sorters (FACS) (Southward and Surette 2002). GFP fluoresces green and requires only the presence of oxygen to mature – no external compound need be added to an organism expressing GFP in order to detect flourescence (Chalfie et al. 1994). GFP does not interfere with the growth of the host; it is brilliant for nondisruptive studies for the study of bacterial communities or other systems. GFP requires live cells to be studied at the single cell level. Several GFP color variants (red, yellow, cyan) are available, which can be easily distinguished from others, allowing simultaneous monitoring of expression (Stuurman et al. 2000; Bloemberg et al. 2000). GFP activity is not influenced by metabolic activities of the organisms. A major disadvantage of GFP is that once formed it seems to be very stable, which in turn renders the protein less valuable of transient gene expression (Bloemberg 2007).

With the discovery and development of AFPs as markers and the development of highly sophisticated flourescence microscopes such as confocal laser scanning microscopes, a new dimension has been created for studying PGPR in their natural environment. Several review articles provide the reader further methods in detail (Bloemberg and Lugtenberg 2004; Chalfie and Kain 2005; Larrainzar et al. 2005; Rediers et al. 2005; Bloemberg and Camacho 2006; Bloemberg 2007).

14.3.6 Lac Z and Lux Gene-Based Reporting Methods

Luminescence-based techniques offer many of the advantages of classical techniques (fluorescent-antibody and selective plating), and no extensive detection of marked cells in soil samples are needed (Sørensen and Nybroe 2006). Stable integration into the bacterial genome was achieved by use of mini-Tn5 delivery vectors (Sørensen and Nybroe 2006). The system permitted the detection of tagged *Rhizobium meliloti* in the presence of more than 10^5 CFU per plate without the use of any selective markers (such as antibiotic resistance genes). No significant

differences in growth rates or soil survival were found between the marked strain and the wild-type strain. Studies of bioluminescent *R. meliloti* also revealed a good correlation between cell biomass and bioluminescence. The firefly luciferase tagging system is an easy, safe, and sensitive method for the detection and enumeration of bacteria in the environment (Cebolla et al. 1993).

A mutant strain, *P. fluorescens* WCS365 with Tn5*lacZ* mutation, colonized roots to a lesser extent than did wild type (de Weger et al. 1987; Simons et al. 1997; Dekkers et al. 1998b; Kozaczuk and Skorupska 2001). Dekkers et al. (1998b) showed that the gene encoding NADH dehydrogenase plays an important role in root colonization. Another gene required for efficient colonization is the *sss* gene, encoding a site-specific recombinase of the lambda integrase family which helps in adapting cells to rhizosphere conditions (Dekkers et al. 1998a). Further, it was hypothesized that a two-component system involving genes *colR* and *colS* plays an important role in the root colonizing ability of *P. fluorescens* strain WCS365 (Dekkers et al. 1998b). A recent study by Miller et al. (2001) has shown that the gene *rpoS* is essential for plant root colonization by *Pseudomonas putida* in a competitive environment. Rainey (1999) identified as many as 20 genes that were induced during root colonization using a novel promoter trapping technology. Chauhan and Nautiyal (2010) have reported *purB* gene that controls rhizosphere colonization in *Pantoea agglomerans*.

The lux operon is a set of genes in *V. fischeri*, a rod-shaped bacterium residing in organisms that live in marine environments. The lux operon is a 9-kb fragment that controls bioluminescence through the catalysis of the enzyme luciferase (Meighen 1993). The bacterial luciferin–luciferase system is encoded by a set of genes labeled the Lux operon. In *V. fischeri*, five such genes (*LuxCDABE*) have been identified as active in the emission of visible light, and two genes (*LuxR* and *LuxI*) are involved in regulating the operon (Urbanczyk et al. 2008). Several external and intrinsic factors appear to induce and inhibit the transcription of this gene set and produce or suppress light emission. Although the lux operon encodes the enzymes necessary for the bacteria to glow, bioluminescence is regulated by autoinduction. An autoinducer is a transcriptional promoter of the enzymes necessary for bioluminescence. Before the glow can occur, a certain concentration of an autoinducer must be present. Thus, in order for bioluminescence to occur, high colony concentrations of *V. fischeri* should be present in the organism (Madigan and Martinko 2006). Isolation of the *lux* genes and the ability to transfer these genes into prokaryotic and eukaryotic organisms have greatly expanded the scope and potential uses of bacterial bioluminescence as a safe, rapid, and sensitive sensor for a wide variety of compounds and metabolic processes. Maize and lettuce seeds were treated with derivatives of all strains marked with *lux* genes for bioluminescence and resistance to kanamycin and rifampin prior to planting in nonsterile Promix and natural soil. The introduced bacterial strains were quantified on roots by dilution plating on antibiotic media together with observation of bioluminescence (Chabot et al. 1996; Darwent et al. 2003).

14.3.7 Luciferase Gene

The eukaryotic luciferase genes, luc from the firefly *Photinus pyralis* (de Wet et al. 1985) and those from the luminous click beetle *Pyrophorus plagiopthalamus* (Wood et al. 1989) also have been expressed successfully in various bacteria (Cebolla et al. 1991; de Wet et al. 1985; Lampinen et al. 1992; Palomares et al. 1991). Each gene codifies a monomeric enzyme that catalyzes the same reaction, involving D-luciferin, ATP, and O_2. Furthermore, comparison of the expression of both types of luciferases in *Bacillus subtilis* showed that bacteria expressing bacterial luciferase suffered a decrease in growth rate with respect to that of the parental strain (Lampinen et al. 1992). This fact may result in a selective disadvantage for these bacteria when competing with indigenous bacteria. In contrast, no significant variation in *B. subtilis* growth was observed upon expression of eukaryotic luciferases. Furthermore, the luciferase activities measured were about threefold higher than that of the bacterial luciferase. Other requirements for tagging of microorganisms include the following: (1) stable inheritance of the engineered tag must be ensured; (2) the risk of transferring the marker gene among ecosystem populations must be avoided; (3) the gene should not be over-expressed; and (4) markers conferring resistance to antibiotics should be avoided (Cebolla et al. 1993; Alvarado et al. 2004).

Stable integration into the bacterial genome was achieved by use of mini-TnS delivery vectors. The procedure developed was applied for tagging of representative Gram-negative bacteria such as *Escherichia coli*, *R. meliloti*, *P. putida*, and *A. tumefaciens*. The system permitted detection of tagged *R. meliloti* in the presence of more than 10^5 CFU per plate without the use of any selective markers (such as antibiotic resistance genes). No significant differences in growth rates or soil survival were found between the marked strain and the wild-type strain. Studies of bioluminescent *R. meliloti* also revealed a good correlation between cell biomass and bioluminescence. The firefly luciferase tagging system is an easy, safe, and sensitive method for the detection and enumeration of bacteria in the environment (Cebolla et al. 1993; Alvarado et al. 2004; Koo et al. 2007).

14.4 Conclusions and Future Prospects

Microorganisms introduced into the environment undergo a wide variety of processes following their introduction including growth, physiological adaptation, conversion to nonculturable cells, physical spread, and gene transfer (Van Elsas et al. 1998). Hence, the application of single methods for microbial detection and for evaluation of their activity in the rhizosphere and risk involved is likely to provide only partial information. Both culture-based and culture-independent approaches have their own advantages and limitations. It is suggested that a polyphasic approach would be most practical for monitoring of microbial inoculant in rhizosphere/bulk soil (Fig. 14.2).

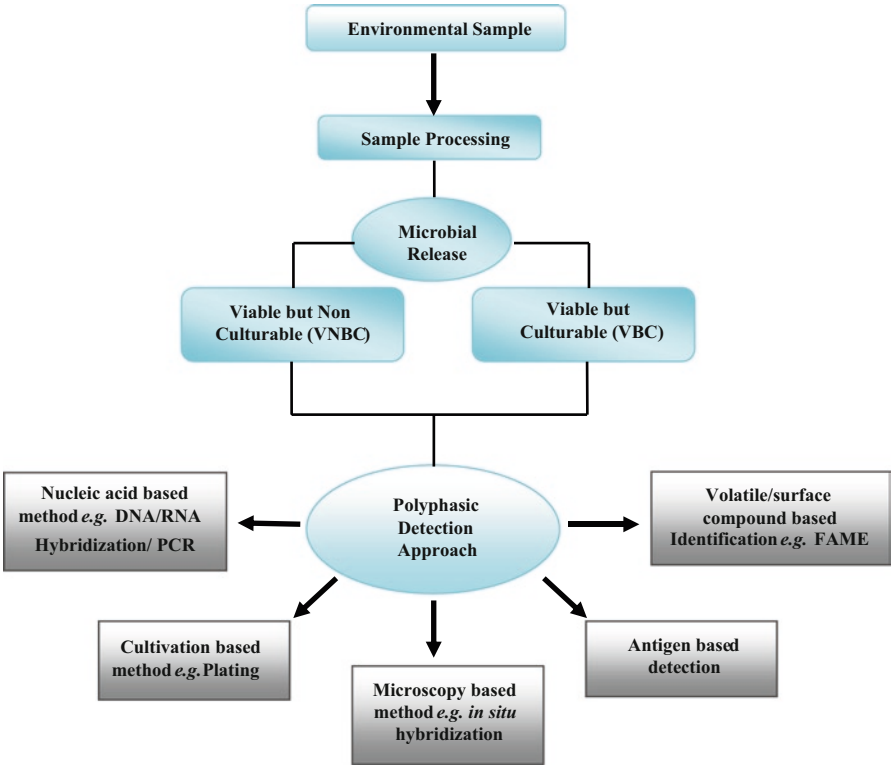

Fig. 14.2 Various approaches to study bacterial diversity from environmental sources

For robust assessment of the fate and effect of released microbial inoculants/ PGPR, it is therefore necessary to use a combination of techniques as the case may depend upon microbe-to-microbe and microbe-to-plant interactions and other environmental factors. Microscopy, cultivation-based and molecular-based techniques should be developed both for genetically modified and unmodified inoculants released into the rhizosphere or the larger environment.

As our understanding of the complex environment of the rhizosphere, of the mechanisms of action of PGPR, and of the practical aspects of inoculant formulation and delivery increase, we can expect to see new PGPR products becoming available. The success of these products will depend on our ability to manage the rhizosphere to enhance survival and competitiveness of these beneficial microorganisms (Bowen and Rovira 1999). Rhizosphere management will require consideration of soil and crop cultural practices as well as inoculant formulation and delivery. Genetic enhancement of PGPR strains to enhance colonization and effectiveness may involve addition of one or more traits associated with plant growth promotion. The use of multistrain inocula of PGPR with known functions is of interest as these formulations may increase consistency in the field. Alternatively, plant growth-promoting microorganisms with multifarious desirable traits and tolerance to environmental conditions are expected to provide improved results

(Ahmad 2006; Ahmad et al. 2008; Imran 2009). They offer the potential to address multiple modes of action, multiple pathogens, and temporal or spatial variability. The application of molecular tools is enhancing our ability to understand and manage the rhizosphere and will lead to new products with improved effectiveness. However, multiple strain-based inoculants will require more careful monitoring for their survival, colonization, and effectiveness in the root zone.

Acknowledgements We are thankful to Dr. S. Hayat for his suggestions and Prof. John Pichtel (Ball State University, USA) for critical reading and improvement of this manuscript.

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Chapter 15
Pesticide Interactions with Soil Microflora: Importance in Bioremediation

Munees Ahemad and Mohammad Saghir Khan

Abstract Modern application of insecticides belonging to different chemical families to boost agricultural productivity has led to their accumulation in soils to levels that affect, directly and indirectly, soil enzyme activities and physiological characteristics of nontarget soil microflora including plant growth-promoting rhizobacteria, and, consequently the performance of crop plants. Various biological strategies can be applied for removing toxic substances, including insecticides, from the environment and are collectively known as bioremediation. Among biological approaches, the use of microbes with degradative ability is considered the most efficient and cost-effective option to clean pesticide-contaminated sites. The present review focuses on the role of naturally occurring rhizosphere microbes involved in degradation or transformation of insecticides.

15.1 Introduction

During cultivation, the majority of economically important crops are infested by insect pests including pod borers, aphids, jassids, and pod flies, which cause a substantial reduction in yields (Mukherjee et al. 2007). In current agronomic operations, pesticides, including insecticides are therefore applied, sometimes excessively or indiscriminately to crops and soils to combat insect problems and consequently to increase productivity of agro-ecosystems. After repeated application, a significant proportion of insecticides may accumulate in upper soil layers (0–10 cm) and exert damaging impacts, not only on the diversity but also on the functionality of ecologically and agronomoically important soil microflora (Das et al. 2005). Subsequently, abnormally high concentrations of insecticides may lead to a considerable loss in soil fertility (Pal et al. 2006).

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_15,

Soil microorganisms, specifically rhizospheric bacteria including plant growth-promoting rhizobacteria (PGPR), facilitate plant growth (Khan et al. 2010) by (i) solubilizing insoluble phosphates; (ii) fixing atmospheric N and transporting it to plants; (iii) facilitating uptake of other plant nutrients; and (iv) synthesizing siderophores and phytohormones (Zaidi et al. 2009). Documented results have, however, shown that insecticide concentrations above recommended values adversely affects both the physiological traits of rhizobacteria and various metabolic activities of plants leading to losses in biomass, symbiotic attributes, nutrient (N and P) uptake, and quantity and quality of seeds of plants (Ahemad and Khan 2010). To circumvent such losses and to reduce dependence on chemical additions to soil, biological methods (i.e., microbes and plants) may be applied to detoxify/remove insecticides from soils.

15.2 Toxicity of Pesticides to Soil Microorganisms and Plants

Soil microbial communities play a critical role in cycling of soil elements and, in turn, affect soil fertility and plant growth. However, beneficial microbial communities are greatly influenced by factors including the application of agrochemicals (Ahemad and Khan 2009), which are applied in modern agricultural practices to attain optimum crop yields. Of the various agrochemicals, pesticides, in particular, include any substance intended for preventing, destroying, repelling, or mitigating a pest. Microorganisms can, however, be tolerant or resistant (e.g., slightly or not affected) to pesticides. If, microorganisms are indeed sensitive, pesticides will interfere with vital metabolic activities of microbes.

The consistent and injudicious use of synthetic pesticides has, nevertheless, become a major threat to beneficial soil microbes (Zahran 1999; Srinivas et al. 2008) and in turn affects the sustainability of agricultural crops. Globally, the greater concern is how to minimize or reduce the effects of pesticides so that the potential negative impacts of these chemicals on microorganisms involved in nutrient cycling, vis-a-vis the productivity of crops could be preserved. In the following section, an attempt is made to highlight the impact of insecticides on soil microflora and agronomic crops.

15.2.1 Insecticidal Impact on Rhizobacteria and Crops

In modern high-input agricultural practices, insecticides belonging to diverse chemical groups (Table 15.1) are used as seed and/or soil treatments to prevent losses due to insect pests. Following application, such insecticides accumulate in soils to undesirable levels and affect, either directly or indirectly, soil enzyme activities and physiological characteristics of nontarget soil microbiota (Table 15.2), thereby leading to loss in productivity of soils. For example, Gundi et al. (2005)

Table 15.1 Examples of insecticides and their mode of action

Mode of action	Chemical type	Examples
Acetylcholinesterase inhibitors	Carbamates	Aldicarb, carbaryl, carbofuran, propoxur, carbosulfan
	Organophosphates	Phorate, chlorpyrifos, omethoate, parathion, methmidophos, malathion, diazinon
GABA-gated chloride channel antagonists	Cyclodienes and other organochlorines (OC)	Lindane, aldrin, endosulfan
	Phenylpyrazoles (fiproles)	Fipronil
Sodium channel modulators	OC	DDT
Acetylcholine receptor antagonists	Neonicotinoids	Imidacloprid, thiamethoxam
Acetylcholine receptor antagonists allosteric	Spinosyns	Spinosad
Voltage-dependent sodium channel blocker	Oxadiazine	Indoxacarb

Adapted from http://www.irac-online.org/

Table 15.2 Impacts of selected insecticides on soil biota

Insecticide	Effects	References
Fipronil and pyriproxyfen	Reduced synthesis of IAA and siderophores in *Rhizobium leguminosarum* and *Mesorhizobium.*	Ahemad and Khan (2009)
Malathion, dimethoate, phorate	Aerobic bacteria, among all groups of microflora, were most adversely affected by all insecticides at normal or four times more the normal rate and phorate was found to be most toxic	Aamil et al. (2005)
Chlorpyrifos	Reduced bacterial numbers, but significantly increased fungal numbers	Pandey and Singh (2004)
Carbofuran	Significant impacts on acetylcholinesterase activity in earthworms	Panda and Sahu (2004)
Dimethoate	Short-term reduction in microarthropod numbers (Collembola), but recovery in numbers after time	Martikainen et al. (1998)
DDT	Reduced bacterial and soil algal populations, but may have increased fungal counts	Megharaj et al. (2000)
Malathion	Short-term impacts on earthworm population	Panda and Sahu (1999)
BHC, phorate, carbofuran, and fenvalerate	Stimulated proliferation of aerobic nonsymbiotic N_2-fixing bacteria and phosphate-solubilizing microorganisms and also their biochemical activities, such as nonsymbiotic N_2-fixing and phosphate-solubilizing capacities, which resulted in greater release of available N (NH_4^+ and NO_3^-) and P in soil	Das and Mukherjee (2000)

observed that a mixture of monocrotophos or quinalphos and cypermethrin had additive, synergistic, and antagonistic effects toward bacteria and fungi and dehydrogenase activity in a black clay soil. Application of monocrotophos, quinalphos, and cypermethrin at different rates used either singly or in combination to soil significantly enhanced proliferation of bacteria and fungi and soil dehydrogenase activity even at the highest level of 25 $\mu g\,g^{-1}$. Antagonistic interactions were, however, more pronounced for soil microflora and dehydrogenase activity when monocrotophos or quinalphos were applied with cypermethrin to soil at the highest rate (25 + 25 μg/g). Synergistic or additive responses, on the other hand, occurred at lower application rates with the same combination of insecticides. Some insecticide-tolerant strains of PGPR are also known. For example, Nazarian and Mousawi (2005) identified strains belonging to *Pseudomonas* and *Flavobacterium* which tolerated concentrations of 2.5, 4, and 8 g/L of guthion, methyl parathion, and dimethoate, respectively. The resistance in these bacteria against such organophosphorus pesticides was probably due to the presence of organophosphorous-degrading plasmids that have the ability to express hydrolytic enzymes.

In a follow-up study, Vasileva and Ilieva (2007) carried out pot trials to determine the effect of pre-sowing treatment of seeds with insecticides promet 400 SK (furathiocarb) at a dose of 3 L/100 kg seeds, and carbodan 35 ST (carbofuran) at 1, 2 and 3 L/100 kg seeds on nodulating ability, nitrate reductase activity, and plastid pigments content of lucerne (cv. obnova). It was found that the insecticides did not depress nodulation; instead, nodule numbers and specific nodulation ability of carbodan 35 ST (3 L/100 kg seeds)-treated plants increased by 23 and 7%, respectively, compared to control. Root length for the variants with pre-sowing treatment of seeds was higher than the control by 7–26%. The variant with carbodan at 2 and 3 L/100 kg seeds and promet increased nitrate reductase activity in roots and that with carbodan at 1 L/100 kg seeds increased nitrate reductase activity in leaves. Total content of plastid pigments increased in all variants with carbodan and was lower than the untreated control in the variant with promet.

Das et al. (2003) investigated the effects of phorate and carbofuran at 1.5 and 1 kg active ingredient per hectare, respectively, on the population and distribution of bacteria, actinomycetes, and fungi as well as the persistence of insecticidal residues in rhizosphere soils of rice (*Oryza sativa* L., variety IR-50). Application of insecticides stimulated populations of bacteria, actinomycetes, and fungi in rhizosphere soils. Stimulation was more pronounced with phorate when compared with carbofuran. Neither insecticide, however, markedly affected *Streptomyces* or *Nocardia* in the rhizosphere soils. Total numbers of *Bacillus*, *Escherichia*, *Flavobacterium*, *Micromionospora*, *Penicillium*, *Aspergillus*, and *Trichoderma* treated with phorate and that of *Bacillus*, *Corynebacterium*, *Flavobacterium*, *Aspergillus*, and *Phytophthora* with carbofuran increased. On the other hand, numbers of *Staphylococcus*, *Micrococcus*, *Fusarium*, *Humicola*, and *Rhizopus* under phorate stress and *Pseudomonas*, *Staphylococcus*, *Micrococcus*, *Klebsiella*, *Fusarium*, *Humicola*, and *Rhizopus* under carbofuran stress were inhibited. Similarly, phorate at 100 and 500 μg/mL substantially reduced IAA production by phosphate-solubilizing bacteria belonging to genera *Serratia*, *Pseudomonas*, and *Bacillus* isolated from

various rhizospheric soils, while P-solubilizing activity of PSB was marginally affected (Wani et al. 2005).

The effect of lindane on microbial populations was analyzed in soil with a history of contamination with various chemicals, including pesticides, by Rodríguez and Toranzos (2003). Soil microcosms were amended with 100 mg lindane/kg soil and microbial populations were monitored for 70 days. A 50% reduction in bacterial cell concentrations in lindane-amended microcosms was observed during the second week of the experiment. Overall, no effect of lindane was observed on the metabolic versatility and genetic diversity in these soils, demonstrating the ability of the bacterial populations to tolerate the stress generated by the addition of pesticides. In another report, pencycuron at field rate (FR), 2FR, and 10FR affected microbial biomass C (MBC), soil ergosterol content, and fluorescein diacetate-hydrolyzing activity (FDHA) differentially. Changes in microbial metabolic quotient (qCO_2) and microbial respiration quotient indicated pencycuron-induced disturbance at 10FR. This study revealed that the metabolically activated microbial population was more suppressed compared to the dormant population (Pal et al. 2006).

The effect of increasing rates of lindane (156.0, 244.0, and 312.0 g/ha), unden (propoxur) (125.0, 187.5, and 250.0 g/ha), dithane and karate (166.6, 209.8, and 333.3 g/ha) on garden eggs (*Solanum melongena*), okra (*Abelmoschus esculentus*), and tomatoes (*Lycopersicum esculentus*) was studied by Glover-Amengor and Tetteh (2008). Yields of garden eggs were suppressed by all rates of lindane. In tomatoes, lower lindane rates increased yields, whereas higher rates suppressed yields below the control. In okra, yields were higher than the control at all lindane levels though yield increments were low. Unden application had the greatest effect on garden egg yields followed by tomatoes, and the least on okra. In the garden egg and tomato treatments, increasing concentrations of unden resulted in decreased yields, though yields were higher in the control plots. The optimum unden rate for garden egg and tomato was U20 (125.0 g/ha). Increasing rates of unden on okra did not have any significant effect. Pesticide application reduced soil fungal populations by 50–70%, while bacterial populations declined by 23–38%. In general, dithane suppressed bacterial populations considerably, whereas karate suppressed fungal populations. Lindane did not have any advantage over other pesticides as it caused the lowest increase in yield. Singh and Singh (2006) evaluated the impacts of diazinon, imidacloprid, and lindane treatments on ammonium-, nitrate-, and nitrite-nitrogen and nitrate reductase enzyme activities in a groundnut field for 3 consecutive years (1997–1999). Diazinon was applied for both seed and soil treatment but imidacloprid and lindane were used for seed treatments only at recommended rates. Diazinon residues persisted for 60 days in both the cases. Average half-lives ($t_{1/2}$) of diazinon were found to be 29.3 and 34.8 days in seed and soil treatments, respectively. In the diazinon seed treatment, NH_4^+, NO_3^-, and NO_2^- nitrogen and nitrate reductase activity were not affected. However, diazinon soil treatment resulted in a significant increase in NH_4^+-N in a 1-day sample which continued until 90 days. Some declines in NO_3^--N were detected from 15 to 60 days. Along with this decline, significant increases in NO_2^--N and nitrate reductase activity were found between 1 and 30 days. Imidacloprid and lindane

persisted for 90 and 120 days with average half-lives of 40.9 and 53.3 days, respectively. Within 90 days, imidacloprid residues decreased by 73.17–82.49%, while lindane residues declined by 78.19–79.86% within 120 days. In imidacloprid seed-treated field, stimulation of NO_3^--N and decline in NH_4^+-N, NO_2^--N, and nitrate reductase activity were observed between 15 and 90 days. However, lindane seed treatment resulted in significant increases in NH_4^+-N, NO_2^--N, and nitrate reductase activity and decline in NO_3^--N between 15 and 90 days. Fox et al. (2007) concluded, via study on interaction of agrochemicals with crop plants, that organochlorine pesticides and other environmental contaminants induce a symbiotic phenotype of inhibited or delayed recruitment of rhizobia bacteria to host plant roots, fewer root nodules produced, lower rates of nitrogenase activity, and a reduction in overall plant yield at time of harvest. Moreover, Evans et al. (1991) reported that omethoate was toxic to some *Rhizobium* strains on direct contact when diffused through agar seeded with these bacteria or mixed in broth cultures containing the bacteria. Omethoate mixed with peat-based legume inoculant and applied to seed of subterranean clover or lucerne significantly reduced number of nodules formed over 3 weeks on seedlings grown in pots of sand, compared with inoculated controls. Rhizobia numbers were reduced markedly by mixing with omethoate. Seed pretreatment with omethoate before inoculation had no effect on nodule number (9–11 weeks after sowing), compared with inoculated controls. In another experiment, Evans et al. (1993) found that the effectiveness of inoculation with *Rhizobium meliloti* was significantly reduced when inoculant was applied to seeds pretreated with omethoate. Nodule numbers and shoot mass per plant were reduced by 6 and 22%, compared to untreated plants.

15.3 Bioremediation

Injudicious use of natural resources has resulted in the contamination of land and water with hazardous substances to a considerable extent in many parts of the world. Contaminated sites continue to be discovered due to increasing urbanization and industrialization. Contaminated sites pose a serious threat to human health and also to the environment. Many biological, physical, and chemical strategies are available to clean up contaminated land or water. Some of the commonly used methods for removing pollutants from soil are presented in Table 15.3.

One of the more promising and cost-effect approaches to address soil contamination problems is bioremediation. Bioremediation is defined as the engineered use of biological agents such as microbes or plants to remove/neutralize/degrade/transform contaminants present in soil, sediments, or water. Bioremediation can take place both in situ and ex situ (Hussain et al. 2009). In situ bioremediation does not require excavation of soils; generally, in situ bioremediation is applied for degradation of pollutants present in saturated soils and groundwater. This method has considerable appeal over other bioremediation strategies due to its low cost and employment of innocuous microflora to biodegrade hazardous chemicals and their derivatives. In this technology, chemotaxis is an important attribute since microbial

Table 15.3 Remediation strategies for contaminated soils

Remediation strategy	Advantages	Disadvantages
Chemical inactivation (immobilization/oxidation)	Rapid	Use of chemicals may be costly and may give rise to added contamination
Incineration	Rapid Reduction in waste volume	High costs of transportation, problems such as combustibility of soil matrix and toxic emissions
In situ vitrification	Reduces leaching and soil volume	High costs to generate required temperature (1,600–2,000°C)
Stabilization/solidification (binding to resins)	Reduces leaching	The cost of binding resins may be very high
Thermal desorption(high temperatures in the absence of oxygen to vaporize or destroy pesticides)	Required less heat than incineration The matrix is not incinerated Reduced emissions	Gaseous emission controls required
Vapor stripping (vacuum is applied to contaminated soil, removing volatile waste)	Generates little wasteFairly cost effective	Only suitable for volatile contaminants
Bio- and phyto-remediation	Low cost and maintenance, environment-friendly, suitable for in situ	Slow compared to incineration/chemical deactivation

Adopted from Atterby et al. (2002)

communities with chemotactic traits migrate toward a site enriched with contaminants (chemoattract). Therefore, by enhancing the chemotactic abilities of cells, in situ bioremediation can be made safer for degrading harmful compounds. The benefits of application of in situ bioremediation include: (i) it does not require excavation of the contaminated soils and is consequently cost-effective and (ii) there is minimal site disruption resulting in simultaneous treatment of soil and groundwater. Conversely, in situ bioremediation also has drawbacks: (i) the method is time-consuming compared to other remedial methods and (ii) the potential efficiency of microbes is subject to seasonal variation and environmental factors. In addition, microorganisms perform better when contaminant molecules provide nutrients and energy for growth. If these conditions are not favorable, the ability of microbes to degrade pollutants is decreased. Alternatively, genetic manipulation of microbes is required to accelerate degradability of the pollutants even though stimulating indigenous microflora is generally preferred.

Ex situ bioremediation processes require excavation of contaminated soils or pumping of groundwater to facilitate microbial degradation. Depending on the state of the contaminant to be removed, ex situ bioremediation is classified as (i) a solid-phase system (including land treatment and soil piles) and (ii) slurry-phase systems

(including solid–liquid suspensions in bioreactors). Solid-phase treatment may be applied to organic wastes (e.g., sewage sludge, animal manures, and agricultural wastes) and problematic wastes (e.g., domestic and industrial hazardous wastes, municipal solid wastes). Solid-phase soil treatment processes include landfarming, soil biopiles, and composting. Slurry-phase bioremediation is a relatively rapid process compared to the other biological treatment processes. In slurry-phase bioremediation, contaminated soil is combined with water and other additives in a large vessel termed a bioreactor and mixed to keep the soil microorganisms in contact with contaminants. Nutrients and oxygen are added, and conditions in the bioreactor are monitored and controlled to create the optimum environment for the microorganisms to degrade the contaminants. When treatment is complete, water is removed from the solids, which are disposed or treated further, if they contain additional pollutants (Sasikumar and Papinazath 2003).

15.3.1 Bioremediation of Insecticides

Numerous processes occur during dissipation of insecticides in the environment, for example: (i) volatilization into the air, (ii) sorption to soil components, (iii) movement in soils through runoff, (iv) leaching into soils, and (v) upward movement in soils through capillary forces (Fig. 15.1) (HCN 1996). Degradation, the principal method for insecticide loss, is the primary process affecting the dynamics of insecticide residues in the environment including persistence in soils. The degradation of insecticides is carried out both by physico-chemical methods

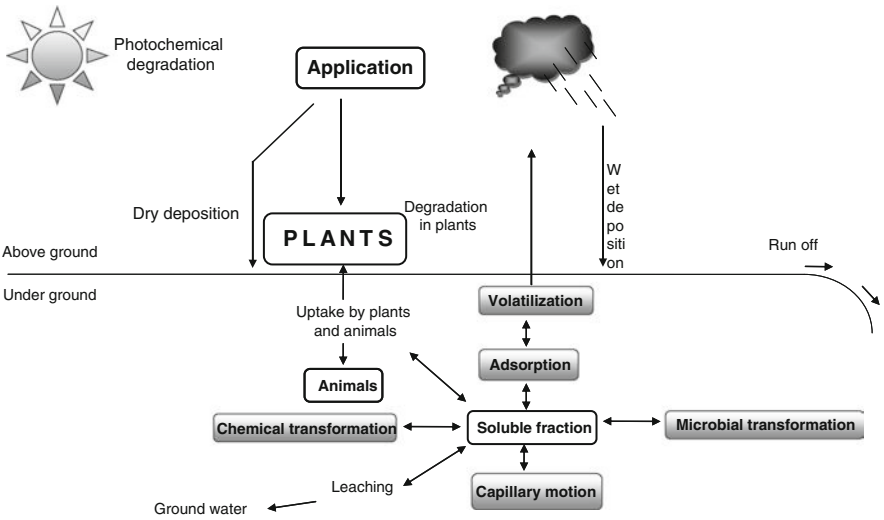

Fig. 15.1 The fate of the pollutants in the environment [modified from HCN (1996)]

and by organisms (microbes and plants). Microbial degradation of insecticides is characterized by enzymes that mediate the splitting of the molecules through different metabolic pathways and is ultimately dependent on the viability, density, and enzymatic activity of soil microorganisms. Furthermore, physico–chemical properties of soils, and types and concentrations of insecticides either applied or previously accumulated in soils, greatly influence degradation rate (Cáceres et al. 2010). In this section, the microbial degradation of insecticides, in particular lindane, chlorpyrifos, and monocrotophos, is discussed primarily because of their toxicity and extensive usage in agricultural production systems.

15.3.1.1 Lindane and Its Isomers

Lindane (1, 2, 3, 4, 5, 6-hexachlorocyclohexane, γ-HCH), a broad-spectrum organochlorine pesticide, is a persistent organic pollutant (POP) and enters soil by direct application, disposal of contaminated waste, or wet/dry deposition from the atmosphere. Immediately following application, HCHs are adsorbed to the soil particles, volatilized to the atmosphere or leached into groundwater, or enter crop plants along with contaminated water. HCHs are strongly adsorbed to soil organic matter (SOM) and, consequently, remain immobile in soils. Nevertheless, under conditions of low SOM and consistent rainfall, lindane, and other HCH isomers pose a significant threat to groundwater (Wauchope et al. 1992). Conventionally, three methods like chemical degradation, physical adsorption, and bioremediation have been reported for the removal of lindane from the contaminated sites. Chemical treatments include the use of microwave irradiation (Salvador et al. 2002), degradation with NaOH-modified sepiolite (Salvador et al. 2002), and addition of hydrogen peroxide (Ahlborg and Thunberg 1980). These treatments involve the use of corrosive chemicals and hence are not eco-friendly. In contrast, physical methods involve thermal desorption and incineration, which provide sufficient degradation but require huge infrastructure and are expensive. In addition, they generate high toxic gases (phosgene). Biological treatments including the use of microbes often called bioremediation, are even though a relatively slow process but are an attractive option due to its inherent eco-friendly characteristics and low cost. In this context, the isolation of microorganisms with lindane degrading potential has confirmed specific strains, which degrade lindane and other HCH isomers (Anupama and Paul 2010) either aerobically or anaerobically. Some strains grow well in media supplemented with HCH as a sole source of C and energy.

Anaerobic Biodegradation Pathway

The first anaerobic lindane-degrading bacterium isolated was *Clostridium sphenoides* UQM780 (MacRae et al. 1969). Subsequently, several other degrading microorganisms were reported, which include genera of *Clostridium*, *Bacillus*, and *Enterobateriaceae* (Kuritz and Wolk 1995; Middeldorp et al. 1996; Boyle et al.

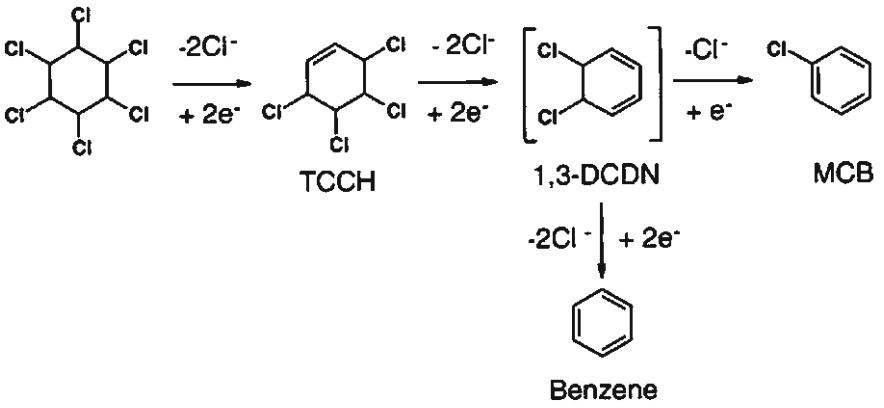

Fig. 15.2 Pathway for anaerobic degradation of HCH [adopted from Middeldorp et al. (1996)]

1999). There is however, inconsistency in results reported for the degradation of different HCH isomers, predominantly owing to varied genera of microorganisms in test soils and degree of tolerance and resilience to contaminants (Moreno and Buitron 2004). Haider and Jagnow (1975) reported that γ-HCH was degraded significantly (up to 90% after 5 days), whereas α- and β-HCH were found to be resistant under both methanogenic and sulfate-reducing conditions (Bachmann et al. 1988). Moreover, the degradation of all four HCH isomers (α-, β-, γ-, and σ-HCH) by mesophilic anaerobic sludges was reported by Buser and Muller (1995) and Quintero et al. (2005). In an anaerobic degradation pathway of HCHs, tetrachlorocyclohexene (TCCH) is identified as an intermediate, even though it is assumed that the primary intermediate was pentachlorocyclohexane (PCCH), which is too unstable to be detected. The complete pathway is shown in Fig. 15.2.

Aerobic Biodegradation Pathway

Complete mineralization of HCH occurs under only aerobic conditions. Microbial aerobic degradation of the four HCH isomers has been observed in both mixed soil cultures (Sahu et al. 1993) and pure cultures (Thomas et al. 1996; Johri et al. 1998). The majority of studies on the determination of an aerobic degradative pathway of lindane and other HCH isomers has concentrated on *Sphingomonas paucimobilis* UT26, a nalidixic acid-resistant mutant of *Sphingomonas* (previously classified as *Pseudomonas*) *paucimobilis* SS86 (Imai et al. 1989; Senoo and Wada 1989). This novel bacterial strain UT26 degrades α-, γ-, and σ-HCH and exploits γ-HCH as a sole source of carbon in the presence of oxygen (Nagasawa et al. 1993). The degradation pathway shown in Fig. 15.3 and involves several enzymes [Lin A (dehydrochlorinase), Lin B (halidohydrolase), Lin C (dehydrogenase), Lin D (reductive dehalogenase), Lin E (dioxygenase), Lin F (maleylacetate reductase), and Lin X (dehydrogenase)] encoded by genes (*linA*, *linB*, *linC*, *linD*, *linE*, *linF*, *linR*, and

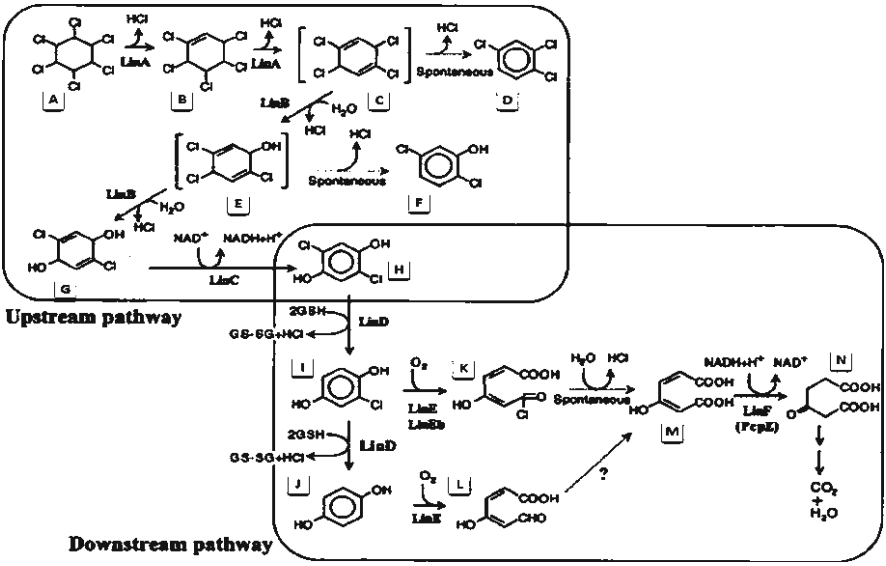

Fig. 15.3 Aerobic pathway of γ-HCH degradation by *S. paucimobilis* UT26. Compounds: *A* γ-HCH, *B* γ-pentachlorocyclohexene, *C* 1,3,4,6-tetrachloro-1,4-cyclohexadiene, *D* 1,2,4-trichlorobenzene, *E* 2,4,5-trichloro-2,5-cyclohexadiene-1-ol, *F* 2,5-dichlorophenol, *G* 2,5-dichloro-2,5-cyclohexadiene-1,4-diol, *H* 2,5-DCHQ, *I* CHQ, *J* HQ, *K* acylchloride, *L* γ-hydroxymuconic semialdehyde, *M* maleylacetate, *N* β-ketoadipate [modified from Endo et al. (2005)]

linX, respectively) and leads to eventual mineralization (Nagata et al. 2006). In addition to these catalytic enzymes, a putative ABC-type transporter system encoded by linKLMN is also essential for the γ-HCH utilization in UT26. After complete genome sequence analysis of UT26, it was found that lin genes for the γ-HCH utilization are dispersed on three large circular replicons of 3.5 Mb, 682 kb, and 191 kb. Nearly identical lin genes were also found in other HCH-degrading bacterial strains, and it has been suggested that the distribution of lin genes is mainly mediated by insertion sequence IS6100 and plasmids. Recently, it was revealed that two dehalogenases, LinA and LinB, have variants with small number of amino acid differences, and they showed dramatic functional differences for the degradation of HCH isomers, indicating these enzymes are still evolving at high speed (Nagata et al. 2007).

In a study, Böltner et al. (2007) isolated four *Sphingomonas* strains, all of which degraded α-, β-, γ-, and δ-HCH. Of these, two strains effectively colonized corn roots reaching a high cell density in cultivated soil and could partly remove γ-HCH. These bacteria, however, performed poorly in unplanted soils. It was suggested that the removal of persistent toxic chemicals can be accelerated by composite application of plants and bacteria, a process generally known as rhizoremediation. Pesce and Wunderlin (2004) reported the aerobic biodegradation of lindane by a consortium of bacteria, *Sphingobacterium spiritivorum*, *Ochrobactrum anthropi*, *Bosea thiooxidans*, and *S. paucimobilis*, from sediment at a polluted site on the Suquia

River, Cordoba, Argentina. The consortia of bacteria showed initial lindane degradation rates of 4.92×10^{-3}, 11.0×10^{-3}, and 34.8×10^{-3} mM h^{-1} when exposed to lindane concentrations of 0.069, 0.137, and 0.412 mM, respectively. Chloride concentration increased during aerobic biodegradation, indicating lindane mineralization. A metabolite identified as γ-2,3,4,5,6-pentachlorocyclohexene appeared during the first 24 h of biodegradation. Pure strains of *B. thiooxidans* and *S. paucimobilis*, however, degraded lindane after 3 days of aerobic incubation. The potential of different enriched bacterial cultures for degrading lindane, methyl parathion (*O*-dimethyl *O*-(4-nitro-phenyl) phosphorothioate) and carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) was assessed under various environmental conditions by Krishna and Philip (2008). Generally, the enriched cultures showed a variable level of degradation and differed with different pesticides. Degradation was more in a facultative anaerobic condition relative to those observed under aerobic condition. In aerobic cometabolic process, the degradation of lindane by lindane enriched cultures was 75 ± 3%, whereas 78 ± 5% of lindane degradation occurred in anaerobic cometabolic process. Degradation of methyl parathion by methyl parathion enriched culture was 87 ± 1% in facultative anaerobic condition. During degradation, many intermediate metabolites were observed, some of which were, however, disappeared after 4–6 weeks of incubation. Interestingly, it was found that the mixture of pesticide-enriched culture was more effective and degraded all the three pesticides more rapidly compared to the sole pesticide-enriched culture. This study suggested that the consortia of bacterial cultures capable of detoxifying the toxicity of multiple pesticides at one time could serve an interesting option for restoring the sites contaminated with multiple pesticides. In addition to bacterial communities, soil also harbors fungi, which are known to degrade lindane very effectively. For example, the degradation of lindane through secretion of certain enzymes has been reported for nonwhite-rot fungus *Conidiobolus* 03-1-56 (Nagpal et al. 2008), white-rot fungi *Cyathus bulleri* and *Phanerochaete sordid* (Singh and Kuhad 2000), and other fungus *Pleurotus ostreatus* (Rigas et al. 2005).

15.3.1.2 Biodegradation of Chlorpyrifos

Chlorpyrifos (*O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate) is a broad-spectrum organophosphate insecticide displaying insecticidal activity against a wide range of insects and other arthropod pests. There are many reports on the degradation of chlorpyrifos by an extensive array of microbial communities inhabiting varied ecological niches and belonging to different genera including *Flavobacterium* and *Escherichia* (Wang et al. 2005; Richinis et al. 1997), *Enterobacter* (Singh et al. 2004), *Arthrobacter* (Mallick et al. 1999), and *Klebsiella* (Ghanem et al. 2007). The importance of microorganisms in the degradation of the organophosphorus insecticide chlorpyrifos during kimchi fermentation was investigated by Cho et al. (2009). Of the 30 mg chlorpyrifos L^{-1} used during the kimchi fermentation, 83.3% of chlorpyrifos was degraded rapidly until day 3, while after

9 days, it was degraded completely. The chlorpyrifos degrading lactic acid bacteria isolated from kimchi fermentation in the presence of 200 mg chlorpyrifos L^{-1} were identified as *Leuconostoc mesenteroides* (WCP907), *Lactobacillus brevis* (WCP902), *Lactobacillus plantarum* (WCP931), and *Lactobacillus sakei* (WCP904). All bacterial strains exhibiting degrading ability, used chlorpyrifos as the sole C and P source. Other insecticides, such as coumaphos, diazinon, parathion, and methyl parathion, were also degraded by the tested strains. In other study, a bacterial strain M-1 isolated from sludge collected from the wastewater treatment pool of a pesticide factory later identified as *Paracoccus* sp. by morphological and biochemical properties and 16 S rDNA sequence analysis was able to degrade 92.47% monocrotophos (100 mg/L) in 24 h. Monocrotophos was used as a sole C source by strain M-1. The biodegradation of monocrotophos was mediated by constitutively expressed cytosolic proteins, which had the greatest activity at pH 8 and 25 C, with its Michaelis–Mentn's constant (K_m) and maximum degradation rate (V_{max}) of 0.29 $\mu mol \times mL^{-1}$ and 682.12 $\mu mol\,(min \times mg)^{-1}$, respectively. The degrading enzyme was sensitive to high temperature, but was active at alkaline conditions (Jia et al. 2007). In a recent investigation, species of *Bacillus* and *Pseudomonas* were found to degrade 75% of chlorpyrifos and phorate and 50% of dichlorvos, methyl parathion, and methomyl within 7 days of incubation. However, dichlorvos and phorate were completely degraded by the end of 14 days and the order of microbial degradation was: phorate > dichlorvos > methyl parathion > chlorpyrifos > methomyl. Qualitative analysis of chlorpyrifos and methyl parathion residues by gas chromatography revealed the formation of one unidentified metabolite in inoculated samples, whereas no metabolite formation was detected in the case of other insecticides-inoculated samples (Madhuri and Rangaswamy 2009). Mallick et al. (1999) reported the rapid degradation of chlorpyrifos, added to a mineral salt medium, or applied to soil as a sole C source, by *Flavobacterium* sp. ATCC 27551 isolated from diazinon-retreated rice fields (Sethunathan and Yoshida 1973). Similarly, an *Arthrobacter* sp. isolated from a flooded soil retreated with methyl parathion has shown chlorpyrifos-degradating ability (Mishra et al. 1992). Moreover, Huang et al. (2000) studied the degradation of chlorpyrifos in poultry and cow-derived effluents and reported that chlorpyrifos was degraded by aerobic microbial processes in animal-derived lagoon effluents. Analysis of the microbial communities involved in the degradation process by denatured gradient gel electrophoresis of PCR-amplified 16 S rRNA genes showed that a single band became dominant in effluents during chlorpyrifos degradation, thus indicating that a single aerobic bacterial population is involved in chlorpyrifos degradation.

Biodegradation of chlorpyrifos is dependent on numerous abiotic factors. Soil pH plays a crucial role in the process. Singh et al. (2003) studied the effects of soil pH on biodegradation of chlorpyrifos in the UK and Australian soils and reported that the dissipation of chlorpyrifos in the UK soils varied at pH values from 4.7 to 8.4 and was mediated by the cometabolic activities of soil microorganisms. A robust bacterial population that utilized chlorpyrifos as a sole source of C was detected in an Australian soil. Transmission and propagation of chlorpyrifos-degrading microorganisms from the Australian soil to UK soils was monitored by

molecular fingerprinting of bacterial 16sRNA genes by PCR-denaturing gradient gel electrophoresis. A heightened ability to biodegrade chlorpyrifos was increased in the UK soils. In addition, only soils with pH≥6.7 were able to maintain this degrading ability 90 days after inoculation. The rate of degradation in chlorpyrifos-degrading bacteria-inoculated soils increased with increasing soil pH from 4.3 to 7.0, but there was no significant difference in degradation rate with pH 7.0–8.4. The degradation rate of chlorpyrifos in acidic soils was slower than in neutral and alkaline soils (Yang et al. 2006). In another study, Singh et al. (2004) reported the enhanced degradation of chlorpyrifos by an *Enterobacter* strain B-14 and found that the strain responsible for enhanced biodegradation of chlorpyrifos showed greatest similarity to *Enterobacter asburiae* based on 16 s rRNA analysis. This strain utilized chlorpyrifos as a sole source of C and P and hydrolyzed it to diethylthiophosphoric acid (DETP) and 3,5,6-trichloro-2-pyridinol (TCP). Further studies with B-14 revealed that the strain possessed a novel phosphotriesterase enzyme system, as the gene coding for this enzyme had a different sequence from the widely studied organophosphate degradative (*opd*) gene (Singh et al. 2004). The authors also concluded that the application of the strain B-14 as bioinoculant in chlorpyrifos-contaminated soil led to substantial increase in the degradation rate of chlorpyrifos than that observed for noninoculated soils. Yang et al. (2005) isolated *Alcaligenes faecalis* DSP3, which has the ability to degrade both chlorpyrifos and TCP. Moreover, Yang et al. (2006) were successful in cloning the *mpd* gene from a chlorpyrifos-degrading bacterium and applying it to bioremediation of contaminated soils. Six chlorpyrifos-degrading bacteria were isolated using chlorpyrifos as the sole source of carbon by enrichment procedure. Their strain, YC-1, showed the highest degrading capability and was putatively identified as the genus *Stenotrophomonas*. The strain YC-1 degraded 100 mg/L chlorpyrifos within 24 h. When chlorpyrifos-degrading strain YC-1 was used as bioinoculant in fumigated and nonfumigated soils, the inoculated soils experienced a more rapid rate of chlorpyrifos degradation compared to the noninoculated control. The initial concentration of 100 mg/kg chlorpyrifos was completely degraded within 15 days. Degradation of chlorpyrifos in control nonfumigated soils (without inoculation) was considerably lower. According to Guha et al. (1997), the *opd* gene for the degradation of chlorpyrifos occurs on plasmids as observed in *Micrococcus* sp. isolated from soil. In contrast, the presence of plasmids was not detected in chlorpyrifos-degrading *Stenotrophomonas* strain YC-1 by the alkali lysis method, which inferred that the *opd* gene was located on the chromosome (Yang et al. 2006). However, both *mpd* and *opd* genes have also been found located variably on chromosome and plasmid. For example, Ajaz et al. (2009) suggested that the biodegradation of chlorpyrifos is mediated by split location of the genes (located on the plasmid and the chromosome) in the *Pseudomonas putida* MAS-1.

In a follow-up study, Li et al. (2007) isolated a highly effective chlorpyrifos-degrading bacterium strain Dsp-2 from the polluted treatment system of a chlorpyrifos manufacturer. This strain identified as *Sphingomonas* sp. by morphological, physiological, biochemical tests, and employing molecular tool (16 S rDNA) could utilize chlorpyrifos as a sole C source for growth by hydrolyzing chlorpyrifos to

3,5,6-trichloro-2-pyridinol (TCP). It could also utilize parathion, parathion-methyl, fenitrothion, and profenofos, but not phoxin and triazophos. Subsequently, the bioremediation ability of this strain was tested under soil environment. When strain Dsp-2 was added to soil treated with 100 mg kg^{-1} chlorpyrifos, it showed a higher degradation rate relative to control soils (without inoculation).The moderate pH, moisture, and inoculum density were found to promote degradation. The gene encoding the chlorpyrifos-hydrolytic enzyme was found as having 99% similarity to *mpd* (a gene encoding the parathion-methyl hydrolyzing enzyme in *Plesiomonas* sp. M6). The hydrolytic efficiency of mpd for chlorpyrifos was significantly greater than the wild-type *mpd* from strain M6.

The degradation of chlorpyrifos is, however, influenced by various factors. In order to assess the impact of variable culture conditions, such as pH, inoculum density, presence of added carbon/nutrient sources, and pesticide concentration, Anwar et al. (2009) conducted an experiment employing *Bacillus pumilus* C2A1 for chlorpyrifos degradation. Chlorpyrifos was utilized by strain C2A1 as the sole source of C and energy as well as it was cometabolized in the presence of glucose, yeast extract, and nutrient broth. Chlorpyrifos was degraded maximally at pH 8.5 and high-inoculum density. Degradation was, however, further enhanced in the presence of other nutrients probably due to high growth on easily metabolizable compounds which in turn increased degradation. The strain C2A1 also showed 90% degradation of TCP (300 mg/L) within 8 days of incubation. In a similar study, Lakshmi et al. (2009) observed that the degradation of chlorpyrifos in soil by three aerobic bacterial consortia, AC, BC, and DC, was greater (50, 56, and 64%, respectively) at 30 days compared to those observed after 21 days (54, 46, and 61%, respectively) growth in basal medium treated with 50 mg chlorpyrifos L^{-1}. *Pseudomonas aeruginosa*, *Bacillus cereus*, *Klebsiella* sp., and *Serratia marscecens* when grown alone in basal medium supplemented with 50 mg chlorpyrifos L^{-1} degraded chlorpyrifos by 84, 84, 81, and 80%, respectively, after 20 days and 92, 60, 56, and 37%, respectively, after 30 days. Formation of 3,5,6-trichloro-2-pyridinol, the major metabolite of chlorpyrifos degradation, was observed during the degradation of chlorpyrifos by *P. aeruginosa*, which disappeared to negligible amounts. This and other associated studies are thus likely to help overcome chlorpyrifos toxicity in contaminated environment.

15.3.1.3 Monocrotophos

The degradation of a widely used organophosphorus insecticide, monocrotophos (dimethyl (E) 1-methyl-2-methylcarbamoyl vinyl phosphate) in two Indian agricultural soils, i.e., a black vertisol and red alfisol, was studied in the laboratory by Gundi and Reddy (2006). The insecticide was applied at two concentrations, 10 and 100 μg g^{-1} soil, under aerobic conditions at 60% water-holding capacity at 28 ± 4°C. The degradation of monocrotophos (MCP) at both concentrations was rapid, accounting for 96–98% of the applied quantity following first-order kinetics with rate constants (k) of 0.0753 and 0.0606 day^{-1} and half-lives ($t_{1/2}$) of 9.2 and

11.4 days, respectively. Degradation of MCP in soils proceeded by hydrolysis, with the formation of *N*-methylacetoacetamide. Even three additions of MCP at 10 μg g^{-1} soil did not result in enhanced degradation. However, there was cumulative accumulation of *N*-methylacetoacetamide in soils pretreated with MCP, i.e., 7–15 μg g^{-1} soil. Both biotic and abiotic factors were involved in MCP degradation. In one study (Bhalerao and Puranik 2009), soil fungi capable of degrading MCP were isolated from various geographical sites. Twenty-five strains were isolated by an enrichment method using MCP as a carbon and phosphorus source. On the basis of MCP tolerance capacity exhibited in gradient agar plate assay, the isolate M-4, identified as *Aspergillus oryzae* ARIFCC 1054, was selected for further studies. The ability of the isolate to mineralize MCP was investigated under different culture conditions. The isolate was found to possess phosphatase activity. The course of the degradation process was studied using high-performance thin layer chromatography (HPTLC) and FTIR analyses. The results suggest that this organism could be used for bioaugmentation of soil contaminated with MCP and for treatment of aqueous wastes.

Degradation of MCP in soils was found to be enhanced by light, moisture (more in flooded soils than in dry loam soils), and type of water (greater in tap water than distilled water) (Dureja 1989). Biodegradation of MCP and other organophosphates by soil bacteria was studied by Rangaswamy and Venkateswarlu (1992). They isolated several strains of *Bacillus* and one isolate of *Azospirillum lipoferum*, which were capable of degrading MCP. Microbial degradation was more pronounced and rapid than chemical decomposition.

Bhadbhade (2001) studied microbial degradation of MCP; microorganisms capable of degrading MCP were isolated from ten soil samples collected from Maharashtra. Among 54 isolates, 74% (32 isolates) were obtained from exposed soils, whereas 26% (22 isolates) were from soils not exposed to MCP. This revealed the predominance and ease in isolating MCP-degrading bacteria from exposed soils. The cultures belonged to the genera *Bacillus* (62%), *Arthrobacter* (22%), *Pseudomonas* (12%), and 2% each to *Planococcus* and *Stomatococcus*. Three cultures identified as *Arthrobacter atrocyaneus*, *Bacillus megaterium*, and *Pseudomonas mendocina* showed 80–90% degradation to MCP at maximum initial concentration of 500 mg/L in synthetic medium within 48 h. The cultures tolerated MCP up to a concentration of 2,500 mg/L and could utilize MCP as a sole source of carbon in synthetic media. The isolates showed maximum degradation of MCP under different environmental conditions; for example, pH values of 7.0–8.0, temperatures of 30–35ºC, MCP concentrations ranging from 100 to 500 mg/L, and an inoculum density of 108–109 cells/mL, in synthetic medium under aerated culture condition in 48 h. The removal of MCP ranged between 77 and 78% (Bhadbhade 2001).

Biodegradation of MCP to phosphates, ammonia, and carbon dioxide was brought about through the formation of intermediate compounds; namely, one unidentified metabolite, methylamine, and volatile fatty acids such as acetic acid or *n*-valeric acid. The isolates were found to exhibit two enzymes, namely phosphatase and esterase, which were involved in the degradation of MCP. The microbial metabolic pathway for the degradation of MCP has been proposed

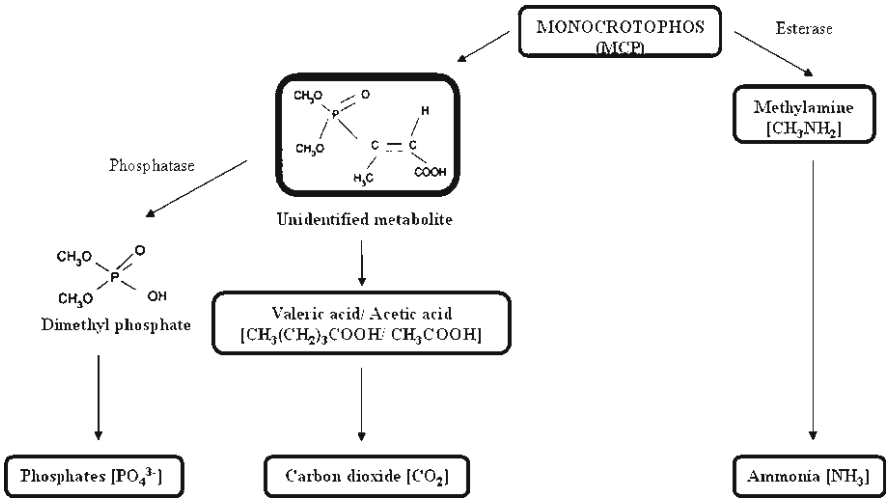

Fig. 15.4 Pathway for degradation of monocrotophos (Bhadbhade et al. 2002a)

based on experimental results (Fig. 15.4) (Bhadbhade et al. 2002a). In addition, MCP degradation genes are now known to be located on plasmids (Bhadbhade et al. 2002b). In a similar study, Subhas and Singh (2003) observed *Pseudomonas aeruginosa* F10B and *Clavibacter michiganense* subsp. *insidiosum* SBL 11 able to degrade technical MCP in shake-flask culture up to 98.9 and 86.9%, respectively, and pure MCP up to 79 and 80%, respectively, within 24 h at 37°C. The optimal concentration of MCP required for the normal growth was 500 ppm. Tris-*p*-nitrophenyl phosphate was found as the most preferred substrate followed by paraoxon. The enzyme involved in the degradation of MCP was phosphotriesterase, which was localized on the membrane-bound fraction of the disrupted cells. The gene responsible for the production of phosphotriesterase (*opd*) in *P. aeruginosa* F10B was plasmid-borne.

15.4 Conclusion

Insecticides in general adversely affect metabolic activities of both soil microflora and crop plants. At recommended dose rates, the toxic effects of insecticides on beneficial activities of rhizobacteria and plant growth parameters are, however, less severe. Rates higher than recommended field rates have been found to decrease nitrogen fixing ability, production of phytohormones, and other regulatory substances in soil microorganisms and photosynthesis, dry biomass accumulation and the general nutrient status of crop plants. Therefore, natural, inexpensive, and eco-friendly microbes endowed with insecticide-degrading potential could be an ecologically sound alternative to detoxify persistent and excessive quantities of residual insecticides in soils.

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Chapter 16
Baculovirus Pesticides: Present State and Future Perspectives

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Abstract Baculoviruses pesticides are ideal tools in integrated pest management programs as they are usually highly specific to their host insects; thus, they do not affect other arthropods including pest predators and parasitoids. They are also safe to vertebrates and plants and to the biosphere. Over 50 baculovirus products have been used against different insect pests worldwide, and all have been produced *in vivo*, mostly on insects reared on artificial diets. However, there are cases of significant viral production in the field by applying a baculovirus against natural populations of the insect host and collecting dead or moribund larvae for further processing into a formulated product. Despite the considerable number of programs worldwide utilizing baculoviruses as biopesticides, their use is still low compared to another biological insecticide based on the bacterium *Bacillus thuringiensis* Berliner. As of the present, there are no programs using *in vitro* commercial production of baculovirus due to several technical limitations, and further developments in this area are much needed. Use of the baculovirus of the velvetbean caterpillar in Brazil has experienced a setback over the past 7 years due to modifications in cultural practices by soybean growers. Slow speed of kill by viral pesticides is a limitation that has led to considerable research effort toward developing faster killing agents through genetic modifications by either deleting or inserting toxin genes from scorpions and spiders into their genomes. However, these GMOs have not been used in practice due to significant resistance by the public to modified baculovirus genomes. Effective public extension services and farmer education toward application of biopesticides are much needed to expand the use of these products worldwide.

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_16,

16.1 Introduction

There are at least 12 viral families associated with insects and other arthropods (Erlandson 2008). The Baculoviridae is the most commonly investigated with regard to its development as a microbial insecticide due to its favorable characteristics such as safety to the environment, humans, other vertebrates, plants, and natural enemies of pests (particularly predators and parasitoids). These viruses are generally highly selective, not affecting other insect species including those that are pests. Consequently, the baculoviruses are ideal control agents to be used in integrated pest management (IPM) programs in agriculture, forests, and pastures. Use of these agents as microbial insecticides was advocated in the 1960s and 1970s (e.g., Ignoffo and Couch 1981; Tanada and Kaya 1993; Cunningham 1995; Moscardi 1999; Szewczyk et al. 2006, 2009).

Baculoviruses have also proven to be extremely valuable tools in biotechnology. The baculovirus–insect cell expression system has become one of the most widely used systems for routine production of recombinant proteins. More recently, baculoviruses have demonstrated the ability to make ideal vectors for a variety of mammalian cell lines and are potential candidates in gene therapy (Kost et al. 2005; Hitchman et al. 2009).

16.2 State of Taxonomy and Biology of Baculoviruses

16.2.1 Taxonomy

Baculoviruses are a large and diverse group of viruses pathogenic to arthropods, primarily insects from the orders Lepidoptera, Hymenoptera, and Diptera. More than 700 baculoviruses have been isolated from invertebrates and reported in the literature (Moscardi 1999; Herniou and Jehle 2007). These viruses occur naturally in insect populations and are normally named for the initial host from which they were isolated. Owing to their high virulence, specificity to insects, and environmental stability, they have been widely used as bioinsecticides for the control of numerous agricultural and forest pests. A number of these viruses have been used to control insects as biological alternatives to chemical pesticides (Moscardi 1999; Szewczyk et al. 2009).

Baculoviruses replicate in the nuclei of infected host cells and possess circular, covalently closed, double-stranded DNA genomes ranging from 80 to 180 kbp in length, encoding for 100–180 proteins (Theilmann et al. 2005). Genomes of more than 50 baculoviruses have been sequenced (NCBI databases) and many have been analyzed and published (van Oers and Vlak 2007; Rohrmann 2008a). These viruses belong to the family Baculoviridae, which is currently subdivided on the basis of phylogenetic evidence and molecular characteristics into four genera: *Alphabaculovirus* (lepidopteran nucleopolyhedrovirus),

Betabaculovirus (lepidopteran granulovirus), *Gammabaculovirus* (hymenopteran nucleopolyhedrovirus), and *Deltabaculovirus* (dipteran nucleopolyhedrovirus). This classification of baculoviruses (Jehle et al. 2006) has been proposed for the 9th International Committee on Taxonomy of Viruses Report (www.ictvonline.org). Lepidopteran NPVs can be further classified into two groups, i.e., I and II. This subdivision has been correlated with the presence of unique envelope fusion proteins, GP64 (Group I) and F (Group II), encoded by viruses from each group (Zanotto et al. 1993; Pearson et al. 2000; Ijkel et al. 2000; Herniou et al. 2001, 2003). Virions of *Alphabaculoviruses* are designated single (S) or multiple (M) depending on the number of nucleocapsids per ODV (occlusion-derived virus), whereas delta- and gammabaculoviruses normally contain a single nucleocapsid per ODV (Volkman et al. 1995; Theilmann et al. 2005).

Baculoviruses exist as two phenotypes, i.e., occlusion-derived virus (ODV) and budded virus (BV), which have a common nucleocapsid structure and carry the same genetic information (Blissard 1996). These virions are produced at different cell locations and times in the infection cycle. Also, they differ with relation to some of their virus-derived proteins, in the composition of their viral membranes, and in their mechanisms of entry into the host cell. BVs are produced in the late phase of infection, obtain their envelope from the cell membrane, and require the fusion protein GP64 (Monsma et al. 1996; Hefferon et al. 1999) or another unrelated protein termed the F protein (Lung et al. 2002; Westenberg et al. 2004) that facilitates systemic infection. This protein forms structures called peplomers at one end of the budded virus particle, but they are not present in ODVs (Monsma et al. 1996), although a number of other proteins are only associated with ODV. Several ODV envelope proteins have been identified as essential for primary infection of midgut cells of insect larvae and others as ODV components whose specific location and function have not yet been determined (Kuzio et al. 1989; Faulkner et al. 1997; Kikhno et al. 2002; Pijlman et al. 2003; Ohkawa et al. 2005; Slavicek and Popham 2005; Fang et al. 2007, 2009; Li et al. 2007). ODVs are produced in the very late phase of the infection when nucleocapsids become enveloped within the nucleus and are subsequently occluded in a protein crystal structure forming the occlusion bodies (OBs).

16.2.2 Viral Life Cycle

In the baculovirus life cycle, ODVs establish primary infection in the midgut and are required for horizontal transmission of baculoviruses between insect hosts. These virions are derived from the nuclear membrane of the insect cell and at a very late time, postinfection, become occluded in a protein matrix, forming paracrystalline structures termed occlusion bodies (OBs). The occlusion bodies are composed mainly of a protein called polyhedrin in NPVs and granulin in GVs that are highly stable and facilitate virus survival and dispersal in the environment (Olszewski and Miller 1997). BVs are highly infectious for insect cells and are capable of spreading

infection from cell to cell both within the insect and in cell culture. These virions have an envelope distinct from ODV that facilitates systemic infection. They acquire their envelopes by budding through the plasma membrane.

The viral life cycle begins when a susceptible host ingests OBs that have been deposited on foliage by a previously infected host, resulting in the release of hundreds of ODVs in the gut. In the host midgut, crystalline polyhedron matrix surrounding the ODVs is dissolved by the alkaline environment. The released ODVs then pass through the peritrophic membrane, attach to the microvilli, and subsequently initiate primary infection of mature columnar epithelial cells within the midgut. Budded virus (BV) produced in these cells initiates secondary infections, spreading throughout the host. The nucleocapsids are released from the endosomes and are transported to the nucleus, where viral transcription, DNA replication, and assembly of progeny nucleocapsids occur, resulting in the production of BV and ODV. In the final stage of infection, most of the nucleocapsids remain in the nucleus and become occluded in a protein matrix to form OBs. Progeny OBs are released upon death and disintegration or liquefaction of the infected insect and subsequently initiate a new round of infection to other hosts. The terminally infected insect can migrate to a higher elevation on the branch of a plant, facilitating dispersal of the occlusion bodies (Kamita et al. 2005a, b; Rohrmann 2008b). The consecutive steps of this complex process of infection are shown in Fig. 16.1.

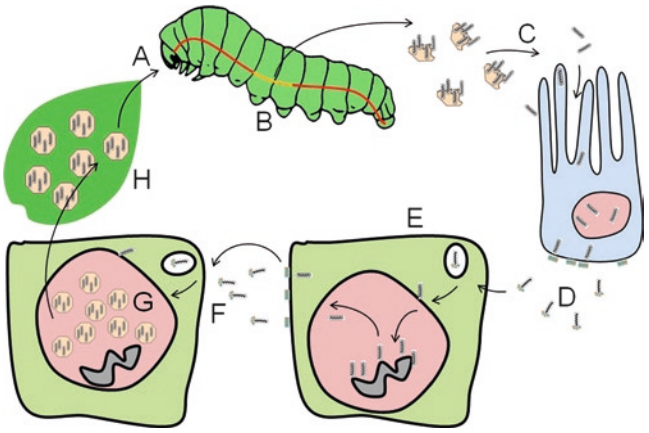

Fig. 16.1 Natural life cycle of baculovirus AcMNPV. Polyhedra are taken orally by the larvae along with plant material (**a**) and are dissolved in the alkaline environment of the midgut (**b**). ODVs are liberated and infect epithelial midgut cells (**c**). The virus replicates, and budded viruses (BVs) are produced (**d**), and they infect other tissues (**e**). After secondary infection (**f**), polyhedra are accumulated (**g**). Finally, the larval body disintegrates, and millions of new polyhedra are released to the environment (**h**). BV – budded form of the virus. ODV – occlusion derived form of the virus

16.2.3 Molecular Biology of Baculoviruses

Baculoviruses are a large group of double-stranded DNA viruses. They infect arthropods and do not replicate in vertebrates, plants, or microorganisms. Though they do not replicate, they may, under special conditions, enter animal cells. This unexpected property has made baculoviruses a valuable tool for studies of transient expression of foreign genes under vertebrate promoters introduced into the baculovirus genome (Boyce and Bucher 1996; Kost et al. 2005).

The baculoviruses have gained immense attention in molecular biology laboratories because they are one among the most versatile genetic engineering tools (for a review see van Oers 2006). The most widely studied baculovirus is the *Autographa californica* nucleopolyhedrovirus (AcMNPV). Our current knowledge about the biology of AcMNPV is, to a large extent, a consequence of the developments of baculovirus-based expression vectors. This system of foreign gene expression has many advantages over other systems, which are as follows:

- A high level of foreign gene expression is usually achieved compared to other eukaryotic expression systems.
- It is possible to express more than one foreign gene.
- The baculovirus genome can accommodate large pieces (around 20 kbp) of foreign DNA.
- Insertion of specific signal sequences in front of a foreign gene often leads to export of the gene product outside of the infected cell.

The circular DNA genome of AcMNPV is surrounded by a small basic protein that neutralizes the negative charge of the DNA. This structure is protected by proteins forming a nucleocapsid. Virions consist of one or more nucleocapsids embedded in a membranous envelope. The genomic circular DNA is infectious in the naked form. As mentioned above, the two morphologically distinct, but genetically identical, viral forms (ODV and BV) are produced at different periods after infection. The occlusion bodies (polyhedra) contain many occlusion-derived virions (ODV) surrounded by a matrix composed mainly of polyhedrin, a major structural protein (Braunagel et al. 2003). It should be stressed here that polyhedrin is produced in large quantities (approx. 30% of total protein mass at the time of host death) but is not needed for transmission of the virus from cell to cell. Polyhedra (OBs) are relatively stable, and protected virions under favorable conditions can survive in the environment for decades. They are large enough to be seen under a light microscope. Under magnification of 1,000×, polyhedra resemble clear, irregular salt crystals.

Recombinant baculoviruses are usually constructed in two steps. Initially, a heterologous gene is introduced into a baculovirus transfer vector. The vector consists of a bacterial replicon of a multicopy plasmid, a selection marker gene, promoter and terminator regions along with flanking baculovirus sequences from a nonessential locus, and a multiple cloning site (or a single unique restriction site) downstream from a viral promoter. When commercial production of a recombinant protein is required, the promoters and the flanking DNA usually originate from one

of the very late genes, either polyhedrin or p10. The latter is another viral gene coding for a protein produced in large quantities very late in the infection. It is the main component of the fibrillar structures, which accumulate in the nucleus and in the cytoplasm of infected cells. For some purposes, e.g., for earlier enhancing of the insecticidal properties of a baculovirus, weaker late but not very late promoters [e.g., basic protein promoter (*p6.9*)] or early promoters (e.g., *ie1*, *p35* or DA26) are sometimes preferred.

Classical methods of recombinant construction are based on the homologous recombination in insect cells as the second step of engineering the recombinant. The baculovirus transfer vector containing foreign DNA and genomic viral DNA are introduced into insect cells where they recombine yielding recombinant virus with an integrated heterologous gene. Many improvements over classical methods (Summers and Smith 1987) for recombinant selection have been made in recent years. Linearization of the baculovirus genome at one or more locations simplifies the construction of recombinant baculoviruses. Linear baculovirus DNA exhibits a greatly reduced infectivity compared to preparations of circular DNA. When a unique restriction site was introduced into the AcMNPV genome, which allows for linearization in the vicinity of the polyhedrin gene, recombinant viruses were obtained at a frequency of about 30% (Kitts et al. 1990). It should be pointed out that recombination between linear genomic DNA and a transfer vector results in circularization of the genome. Therefore, even though the titer of recombinants per transfection is similar to that of the normal cotransfections with circular genomic DNA, the percentage of recombinants is greatly increased because the background of nonrecombinants originating from linear DNA is greatly reduced. Further developments of the above method increased the percentage of recombinant viruses to almost 100% (Kitts and Possee 1993).

Many laboratories specializing in the production of recombinant proteins routinely use the Bac-to-Bac expression system for constructing baculovirus recombinants (Luckov et al. 1993). The diagram shown in Fig. 16.2 outlines the key steps of recombinant construction. A bacmid (baculovirus shuttle vector) is an engineered low-copy bacterial plasmid (F1 derivative) containing the complete baculovirus genome. The gene of interest is cloned into another small plasmid (e.g., pFastBac) downstream of the polyhedrin promoter. This plasmid also contains two transposable elements flanking the gene of interest and a gentamycin-resistance gene. The donor plasmid is used to transform special bacterial strains containing the baculovirus genome. These bacteria also contain a plasmid coding for the enzyme transposase that catalyzes transposition between the transposable elements engineered in the donor plasmid and those engineered in the viral genome. As a result, the bacmid containing the gene of interest is obtained and can be visually selected because of the presence of an additional *LacZ* marker gene within the viral genome. After verifying the presence of the gene of interest in the baculovirus genome, recombinant bacmid preparations from bacteria are used to transfect insect cells. Following transfection, viable recombinant baculovirus should be budding into the culture medium within 2–3 days posttransfection.

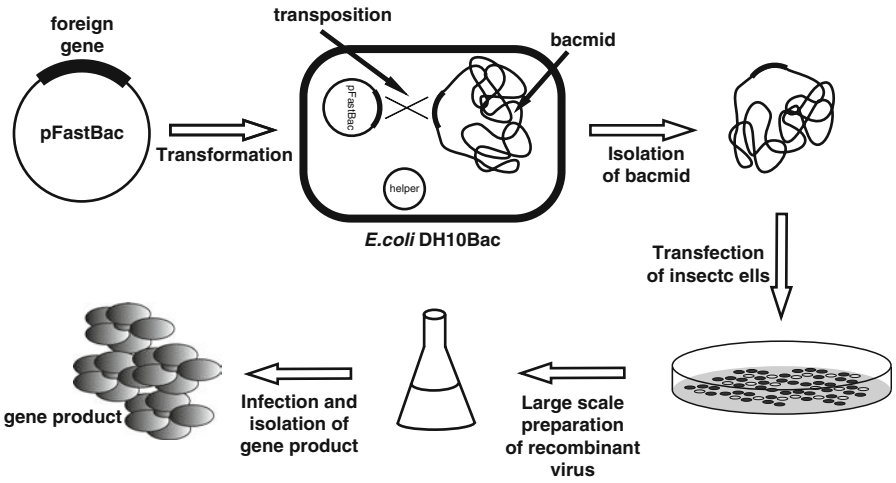

Fig. 16.2 Schematic diagram of Bac-to-Bac expression system. The foreign gene is inserted into pFastBac vector, and the plasmid is introduced into specially engineered *Escherichia coli* strain containing Bacmid – large low-copy plasmid and a helper plasmid allowing for site-specific transposition. The recombinant Bacmid is isolated and transfected into cultured insect cells. The transfected cells are then lysed to yield recombinant baculovirus used later for large-scale preparation of the baculovirus and for the production of the recombinant protein

A few hundred insect cell lines that can potentially be used for *in vitro* propagation of baculoviruses are known. A few that support the growth of AcMNPV were obtained from two parental organisms, *Spodoptera frugiperda* and *Trichoplusia ni* (Lepidoptera: Noctuidae). The most widely used is Sf9, which grows well in suspension (Summers and Smith 1987). BTI-Tn5B1-4 derived from *T. ni*, known as High Five cells, has also been used for viral growth (Granados et al. 1994). Cell lines that can be used for propagation of *Lymantria dispar* nucleopolyhedrosis virus (LdMNPV), *Helicoverpa zea* nucleopolyhedrosis virus (HzSNPV), *Bombyx mori* nucleopolyhedrovirus (BmSNPV), *Anticarsia gemmatalis* nucleopolyhedrovirus (AgMNPV), and a few other baculoviruses are also currently available.

The baculovirus expression system is widely used for production of glycoproteins with therapeutic potential for humans and animals. Most posttranslational modifications of these proteins are the same as in mammalian cells. However, N-glycosylation of proteins in mammalian cells is more complex than that in insect cells. In the latter, N-glycans with terminal mannose residues are produced, in contrast to sialic acid-terminated glycans in vertebrate cells. In most cases, the extent and quality of glycosylation in insect cells are sufficient for preservation of biological activities of glycoproteins, and such insect-derived glycoproteins fulfill the requirements for a potential therapeutic agent. In rare cases, when the role of glycan chains in preservation of biological activity is very high, it is possible to use “humanized” insect cell lines (Harrison and Jarvis 2006), which are genetically engineered to produce the required vertebrate-type complex N-glycans with terminal sialic acids.

16.3 Baculovirus Production Technology

16.3.1 In Vivo Production

At present, commercial production of baculoviruses has been carried out only *in vivo*, either by applying the virus against the host insect in the field and collecting diseased or dead larvae or by producing the target insect in the laboratory on an artificial diet and contaminating the diet with a baculovirus for further collection of virus-killed insects. The latter is the most commonly used method for producing baculoviruses in different countries. Both methods have been used successfully for commercial production of the *Anticarsia gemmatalis Alphabaculovirus* (AgMNPV) in Brazil (Moscardi 1999, 2007). For some insects, there are no available artificial diets, and therefore, commercial production of baculoviruses of these insects is generally too difficult or impossible under laboratory conditions due to dependency of host plant leaves for viral inoculation. On the other hand, field production of baculovirus agents is viable, resulting in a product of lower cost (Moscardi 1999). However, field production is difficult when liquefaction of the insect body is very intense, as, for instance, in larvae infected by *Spodoptera* spp. baculoviruses, making it almost impossible to collect dead larvae. In this case, live larvae must be collected close to death when the body has not yet ruptured. These larvae may, however, contain less virus than would dead larvae. It is known that two viral enzymes, chitinase and cathepsin, are important in the process of cuticle disintegration and liquefaction of the insect body, which are common among species of Lepidoptera. Among natural isolates of a same baculovirus, it is possible to find a few which lack these enzymes, thus facilitating field and laboratory production. The commercial field and laboratory production of the AgMNPV are discussed in Sects. 16.4.1.2 and 16.4.1.3, to exemplify details of both production methods.

16.3.2 In Vitro Production

Baculovirus production in insect cell cultures offers advantages over *in vivo* multiplication for being a controllable, sterile, highly pure product yield process, besides the fact that hundreds of cell lines have already been established. The process of baculovirus production for agricultural pest control needs to be efficient, with competitive cost, leading to a final product that is highly pathogenic to the target pest. There is a strong limitation for *in vitro* production, however, since successive passages of the virus in cell culture result in genetic alterations, leading to loss of virulence (Krell 1996; Rhodes 1996). In laboratory culture, production of occlusion-derived virions (ODV) is not necessary for survival of the virus. The budded virus (BV) particle is the form used for cell-to-cell transmission in cell culture. The main protein of the BV particle is the GP64 (Blissard 1996). During infection, this

glycoprotein is essential for virus budding and is responsible for entrance of the virus into the next host cell (Monsma et al. 1996). Various culture conditions are known to influence infection of lepidopteran cells by baculoviruses and include temperature, pH, dissolved oxygen concentration, osmolality, and nutrient composition of the culture medium. Most lepidopteran cells proliferate optimally at temperatures between 25 and 28°C with an optimum pH of 6.2. Insect cells present several comparative advantages to mammalian cells such as ease of culture, higher tolerance to osmolality and by-product concentration, and higher expression levels when infected with a recombinant baculovirus (Agathos 1996; Ikonomou et al. 2003).

In vitro production remains an important requirement from a commercial perspective for the use of baculoviruses as insecticides. One of the most important effects of the viral passage is the change from the parental, many polyhedra per cell (MP) phenotype, to the few polyhedra per cell (FP) phenotype (Fraser and Hink 1982; Fraser et al. 1983; Pedrini et al. 2004; Rezende et al. 2009; Slavicek et al. 1996). A key problem associated with the passage effect is the reduced occlusion and loss of virulence of the occluded virus. Frequent mutations have been identified within a specific region in the Few Polyhedra mutants (FP) that contains the 25k *fp* locus. This gene encodes a 25-kDa protein that is essential for virion occlusion and polyhedron formation (Chakraborty and Reid 1999; Harrison and Summers 1995; Lua et al. 2002; Pedrini et al. 2004; Slavicek et al. 1996). Another type of mutant generated during serial passage of baculovirus is the formation of Defective Interfering Particles (DIPs). These mutants have lost the ability to be replicated in the host cell without the aid of a helper virus, and large sizes portions of their genome are usually deleted (Bangham and Kirkwood 1990; Kool et al. 1991; Pijlman et al. 2001).

Another challenge for *in vitro* production of baculovirus is the requirement for a highly productive insect cell line (Jem et al. 1997) and a highly productive culture medium (Chakraborty et al. 1999). Many cell lines are available for production purposes and are derived from various sources, thus exhibiting a wide variety of growth and production characteristics. Careful screening or formulation of media must be performed for a particular virus isolate–cell line combination, as different media can greatly affect polyhedra yields (Pedrini et al. 2006). Recently, a new strategy for *in vitro* production has been proposed based on Many Polyhedra (MP) variants. These are clones selected using the plaque assay technique after several passages of the virus in cell culture. MPs maintain the wild-type features such as formation of many polyhedra in the cell nucleus and Budded Virus high titer (Slavicek et al. 2001; Pedrini et al. 2005), which allow them, in principle, to be competitive with the population of Few Polyhedra mutants accumulated in cell culture. The investigation of factors associated with loss of genetic stability and the use of new strategies such as isolation of more stable variants (MP), as well as the reduction of cost of cell culture medium components, is an important requirement for process optimization of *in vitro* baculovirus production.

16.4 Use of Baculoviruses for Pest Control

Since the comprehensive review by Moscardi (1999) on use of baculoviruses for control of Lepidoptera, other works have been published on the state of virus utilization against insect pests of agricultural, forest, and vegetable production systems (e.g., Copping and Menn 2000; Szewczyk et al. 2006, 2009; Souza et al. 2007; Erlandson 2008). In this chapter, we focus on the most important programs worldwide, with emphasis on those aspects that benefit or limit use of these agents in IPM programs. The use of the AgMNPV in Brazil is presented as a case study to discuss how a very successful program (the most important one worldwide) experienced a serious setback over the past 6 years.

The main baculoviruses that have been or are currently being utilized are depicted in Table 16.1. In Latin America, the AgMNPV is the most commonly used biological product to control *A. gemmatalis* in soybean (*Glycine max*). This virus was used in about 2.0 million hectares during the 2003/2004 growing season in Brazil, representing approximately 10% of the soybean cultivated area in the country. It has also been used in Argentina, Colombia, Bolivia, Paraguay, and Mexico (Moscardi 1999, 2007; Sosa-Gómez et al. 2008). Another virus that is presently used in Brazil is the nucleopolyhedrosis of the poplar moth, *Condylorrhiza vestigialis*. This virus has been produced on insects reared on an artificial diet. The primary objective of its application is the treatment of 2,000 ha/year, which represents the infested area among 5,500 ha of poplar (*Populus* spp.) plantations in south Brazil (Sosa-Gómez et al. 2008). In Peru, a granulovirus has been developed to control larval populations of the potato tuber moth, *Phthorimaea operculella*, in field and stored potatoes, by the initiative of the International Potato Center (CIP) (Raman et al. 1992). This virus has also been used in Bolivia, Colombia, and Ecuador (Moscardi 1999; Sosa-Gómez et al. 2008). Presently, another baculovirus used in Latin America is the *Erinnyis ello* GV in Colombia, which has replaced chemical insecticides in sites of endemic occurrence of the insect (Bellotti 1999, Bellotti, pers. communication). Apparently, there are no significant programs using entomopathogenic viruses in Cuba, since there are no reports in the literature, and contacts with Cuban researchers on use of baculoviruses for pest control have not been acknowledged.

The genera *Heliothis* and *Helicoverpa* represent key pests of several annual crops and vegetables worldwide (Ignoffo and Couch 1981; Cunningham 1995), responsible for losses of millions of dollars annually. An NPV of *H. zea* was developed in the 1960s and registered in 1975 in the USA (Ignoffo and Couch 1981), representing an important breakthrough in virus use. Elcar™, developed by Sandoz, was the first viral insecticide registered in the USA for use in cotton. The HzSNPV has a relatively broad range, infecting other species belonging to the genera *Helicoverpa* and *Heliothis*. An HzSNPV formulated product registered as GemStar™ has been used to control *Helicoverpa armigera* on cotton in Australia. Locally obtained isolates of *H. armigera* SNPV have also been produced and applied to cotton, soybean, pigeon pea, maize, and tomato crops in China, India, and Australia

Table 16.1 Main examples of baculoviruses developed as microbial insecticides

Host insect	Baculovirus	Product name(s)	Target crop(s)	Key References
Adoxophyes honmai	GV	–	Tea	Nishi and Nonaka (1996) and Nakai (pers. com.)
Adoxophyes orana	GV	Capex 2	Apple	Cunningham (1995) and Erlandson (2008)
Anticarsia gemmatalis	NPV	Baculovirus Nitral, Coopervirus, Baculovirus AEE	Soybean	Moscardi (1999, 2007)
Buzura suppressaria	NPV	–	Tea, tung oil tree	Sun and Peng (2007)
Cydia pomonella	GV	Madex, Virosoft, Capex, Carpovirusine, Granupon, Virin Cyap, Cyd-X	Apple, pears	Vincent et al. (2007)
Helicoverpa zea	NPV	Elcar, GemStar	Cotton, vegetables	Moscardi (1999) and Erlandson (2008)
	NPV	Gemstar, others	Cotton	Moscardi (1999) and Erlandson (2008)
	NPV	Elcar, GemStar		
Homona magnanima	GV	–	Tea	Nishi and Nonaka (1996) and Nakai (pers. com.)
Lymantria dispar	NPV	Gypcheck	Forestry	Erlandson (2008)
Neodiprion abietis	NPV	Abietiv	Balsam fir	Lucarotti et al. (2007)
Neodiprion lecontei	NPV	Lecontvirus	Pine	Erlandson (2008)
Neodiprion sertifer	NPV	Neocheck-S, Virox	Forestry	Erlandson (2008)
Orgyia pseudotsugata	NPV	TM Biocontrol	Douglas fir	Cunningham (1995) and Erlandson (2008)
Phthorimaea operculella	GV	PTM Baculovirus, Matapol	Field and stored potatoes	Moscardi (1999) and Sosa-Gómez et al. (2008)
Spodoptera exigua	NPV	Spod-X, Ness-A, Ness-E	Horticulture, glasshouse, and field crops	Erlandson (2008)

(Zhang et al. 1995; Sun and Peng 2007; Erlandson 2008; Srinivasa et al. 2008). The potential application of the NPVs of *H. zea* and *H. armigera* is enormous, as *H. zea* and *H. virescens* in the Americas and *H. armigera* in Africa, Asia, and Australasia cause severe losses to several crops and vegetables. In China, NPVs of *H. armigera* are used on over 100,000 ha annually involving at least 12 HaSNPV producers (Sun and Peng 2007).

Another insect genus that causes a severe economic impact on food production is the *Spodoptera* complex, including *S. frugiperda*, *S. exigua*, *S. littoralis*, and *S. litura.* In Brazil, an indigenous isolate of *S. frugiperda* NPV (SfNPV) was used to control the insect in maize and was applied to 20,000 ha/year (Valicente and Cruz 1991; Moscardi 1999). Owing to difficulties and high cost of SfNPV production by the Brazilian Organization of Agricultural Research (Embrapa), a government research institution, this program has been discontinued temporarily – at present, no SfNPV product is available to maize farmers. Presently, a *S. exigua* NPV, under different trade names, has been used to control this species on vegetable crops in the USA, Europe, China, and Thailand. Also, an NPV of *S. litura* is used in China, India, and Thailand (Sun and Peng 2007; Erlandson 2008; Kumari and Singh 2009; Szewczyk et al. 2009).

One of the most important successes in commercial production and use of a baculovirus in Europe may be the coddling moth, *Cydia pomonella*, GV (CpGV) for use in orchards, particularly apples and pears. *The CpGV* has been produced under different trade names (Table 16.1) and has been used in Argentina, Canada, France, Germany, Russia, and Switzerland, among other countries (Moscardi 1999; Arthurs and Lacey 2004; Vincent et al. 2007; Erlandson 2008; Kutinkova et al. 2008). The product Madex® (Andermatt BIOCONTROL), initially developed to support organic fruit producers in Europe, is now produced for application on over 250,000 ha units/year (Vincent et al. 2007). Considering application of other trade names of the CpGV, this may be the most important worldwide viral insecticide currently applied in terms of treated area.

Other important viruses that are currently employed to control insects include the tea tortricids *Adoxophyes honmai* and *Homona magnanima* granuloviruses (GV) in Japan. From 1990 to 1993, five GV production facilities were established in Kagoshima County. These facilities were government-subsidized but operated by a tea growers cooperative. The area sprayed with GVs comprised 5,850 ha in Kagoshima in 1995, equivalent to 80% of all the tea fields in the prefecture (Nishi and Nonaka 1996). The GVs of *H. magnanima* and *A. honmai* were registered in 2003 and produced by Arysta LifeScience Corporation (M. Nakai, pers. communication); however, the use of GVs has declined. One reason for the reduction in use of GVs in Japanese tea fields is the changing pattern of occurrence of other pests. Mulberry scale, for example, has been increasing recently, and chemical treatment is required to control this insect at the same time GVs are sprayed. However, the spray also kills *H. magnanima* and *A. honmai*. Furthermore, GVs have been applied in Kagoshima for more than 10 years, and the populations of *H. magnanima* and *A. honmai* have been reduced (Nakamura 2003). In China, approximately 12 baculoviruses have been authorized as commercial insecticides, including *H. armigera*

NPV (cotton, pepper, tobacco) (which is the most heavily used virus in the country), *S. litura* NPV (vegetables), *S. exigua* NPV (vegetables), *Buzura suppressaria* NPV (tea), and *Pieris rapae* GV, and *Plutella xylostella* GV (vegetables) (Sun and Peng 2007). Use of baculoviruses in China is the greatest worldwide, regarding the number of viruses being registered for insect control. Sun and Peng (2007) also report a Cypovirus (CPV) produced in China for control of *Dendrolimus punctatus*, an insect pest of pine forests.

In forest systems, especially in temperate regions, defoliating larvae of Lepidoptera and Hymenoptera are often significant pests. A *Lymantria dispar* (Lep.: Lymantriidae) MNPV has been developed since the 1980s as a viral insecticide under the trade names Gypcheck and Disparvirus, among others (Moscardi 1999; Reardon et al. 1996; Erlandson 2008; Szewczyk et al. 2009). Also, NPVs of hymenopterans such as *Neodiprion sertifer*, *N. abietis*, and *Diprion pini* (Diprionidae) have been developed as bioinsecticides (Lucarotti et al. 2007; Erlandson 2008; Szewczyk et al. 2009). Forest ecosystems tend to be more stable than agricultural systems, allowing for natural or applied baculoviruses to remain in the environment for long periods of time.

16.4.1 Use of the Alphabaculovirus of Anticarsia gemmatalis (AgMNPV) in Brazil and Latin America: A Case Study

The virus AgMNPV serves as the most important testament that baculoviruses are a viable insect control strategy in the context of an IPM program. Conversely, when an IPM program is not available or not functioning adequately, use of a baculovirus may not succeed. The evolution of the AgMNPV use may serve as an example (Moscardi 1999, 2007), as its applications in soybean in Brazil reached approximately 2.0 million hectares in 2003/2004 season. However, due to changes in agricultural practices by soybean growers, use of the AgMNPV experienced a sharp decline in the last 7 years. This program is summarized and discussed in the sequence.

16.4.1.1 Historical Perspective

The AgMNPV program was established after a pilot phase, conducted during 1980–1982 in farmers' fields in various regions in south Brazil. The virus was found to be efficient for control of *A. gemmatalis* with only one application, compared to 1.8 insecticide applications in areas conducted according to farmer's perceptions (Moscardi 1999, 2007). Implementation of the program for AgMNPV use began in the 1982/1983 soybean season, when approx. 2,000 ha of soybean were treated. Initially, small amounts of the virus were produced in *A. gemmatalis* larvae reared on an artificial diet at Embrapa Soybean facilities (Londrina, PR). At that time, frozen killed larvae were distributed to extension

officers for treatment of demonstration plots and virus production in the field, which provided inocula to treat other areas in the same season or to collect and store dead larvae for the subsequent season. AgMNPV usage gained momentum with the development of a wettable powder formulation of the virus in 1986 (Moscardi 1989, 1999).

Another important step for consolidation of the AgMNPV program was the legal agreement between Embrapa and five private companies in 1990. Through these agreements Embrapa would transfer all the technology for AgMNPV production, formulation, and quality control of production batches. The products based on the AgMNPV of each company were registered according to Brazilian policies for registration of plant protection insecticides (Moscardi and Sosa-Gómez 1996). With the commercialization of the AgMNPV by private companies, the use of this virus increased from about one million hectares in 1990 to approximately 1.5 million hectares in 1995, with most of the production occurring in the field during each soybean season. Peak AgMNPV use occurred in the 2003/2004 season, when approximately two million hectares of soybeans were treated with the virus. Afterward, the use of this agent declined sharply because of changes in farmers' procedures to control pests in soybean (Fig. 16.3), which are discussed below. In addition to the early efforts by Embrapa to develop and improve, both technically and economically, *in vivo* procedures for AgMNPV production under controlled laboratory conditions, two of the companies also attempted to develop production methodologies. One of them (Geratec) produced about 150,000 ha-equivalent of the virus per year in the early 1990s. However, owing to the high cost of labor, disposable rearing containers, and components of the insect artificial diet, laboratory production of the virus was discontinued by both companies. On the other hand, AgMNPV field production became widely

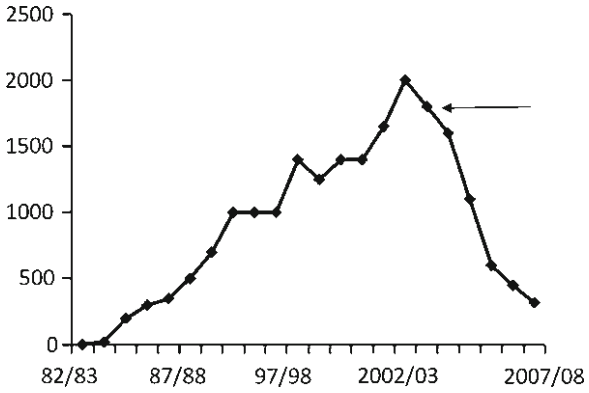

Fig. 16.3 Evolution of the use of the Alphabaculovius of *Anticarsia gemmatalis* (AgMNPV). In Brazil. *Y*-axis = Number of treated hectares (×1,000); *X*-axis = Soybean seasons since 1982/1983. The *arrow* indicates when the soybean integrated program in Brazil started to have a setback, making difficult the use of a very specific baculovirus, as discussed in the paper

adopted by all participating companies as the best available method to obtain large quantities of virus-killed larvae at low cost (Moscardi 1999, 2007). Both methods (field and laboratory) of AgMNPV production are discussed in this sequence. This virus was also used in Argentina and is currently being used in Paraguay and Mexico (Sosa-Gómez et al. 2008).

16.4.1.2 AgMNPV Field Production

Field production of the virus became a major enterprise during the 1980s and 1990s, involving small companies that specialized in marketing AgMNPV-killed caterpillars to private companies that registered the virus for commercialization (Moscardi 1999, 2007). Growers' fields were contracted and pest control in their fields was implemented by the AgMNPV producers. Usually, about three fields are sprayed per day during the prevalence of *A. gemmatalis* larvae in soybeans (December and January). Before collection, fields that are sprayed each day with the AgMNPV are inspected at the 6th or 7th day postapplication for selection of those that would yield the highest number of dead larvae per *hectare*. Peak collection occurs from the 8th to the 10th day after virus application and may involve 200–300 "larval pickers" per day, requiring ten buses to transport them to the fields. In a single day, production at one collection site could reach 600 kg of AgMNPV-killed larvae, enough for treatment of 30,000 ha. To emphasize the importance of production in the field, during the 2002/2003 season, approximately 45 metric tons of AgMNPV-killed caterpillars were collected and sold to the private companies at about US$ 10–12/kg, representing about two million hectare-equivalents of the biological insecticide to be applied in the subsequent soybean season.

Despite its value for producing high quantities of AgMNPV at low cost, field production presented problems that restricted the expansion of its use or affected the quality of the end-product, for example, (1) yearly production was too dependent on natural incidence of the host insect, which may occur in low numbers in certain seasons, thus reducing AgMNPV yield and resulting in variable quantities of the biological insecticide to growers from season to season; (2) quality of field-collected AgMNPV-killed larvae decreased owing to change in collection procedures to attend the high demand by the private companies that registered their AgMNPV commercial products. A key problem was that collection of dead larvae in the field shifted from handpicking to shaking plants over pieces of cloth placed over the ground in between soybean rows. This shift resulted in collection of dead larvae, live host larvae (containing low amounts of virus), larvae from other lepidopteran species, other insects (stink bugs, beetles, etc.), and leaves, which resulted in material with higher amount of extraneous organic matter other than the AgMNPV-killed larvae. While the handpicking method resulted in an average 50 ha equivalent of the virus per kg, the newer procedure resulted in an average 30–35 ha equivalent of the virus per kg. Because of the higher amount of extraneous organic matter, standard procedures for homogenization and formulation had to

be modified. Additionally, higher amounts of organic matter in the final product led to nozzle clogging and decreased efficiency of the product in the field. Therefore, commercial laboratory production of the AgMNPV became a requirement to improve quality. To achieve this goal, research was conducted at Embrapa Soybean to carry out the necessary improvements to make commercial laboratory production viable.

16.4.1.3 AgMNPV Commercial Laboratory Production: A Breakthrough

Improvements in the AgMNPV laboratory production procedures up to 1997 (Moscardi et al. 1997) served as a starting point for a PhD study (Santos 2003) aimed at removing the most important bottlenecks related to commercial production of AgMNPV. Among various aspects studied (ingredients of the insect diet, rearing conditions, containers, virus dosage, larval size at inoculation, and number of larvae per container), significant progress in AgMNPV production was attained as a result of these studies. Cost for the artificial diet was reduced by approximately 85% through substitution of agar by another jellifying agent, and through the reduction of casein content by 50%. With these new procedures, the cost of AgMNPV-killed larvae was approximately US$ 0.42 to treat one ha, as compared with US$ 0.30 for those collected in the field (Santos 2003). Considering the much higher quality of laboratory-produced AgMNPV plus the cost involved, the product generated from laboratory production could be offered at a lower cost than that of chemical insecticides to control *A. gemmatalis*.

In May 2003, a private company (Coodetec) established a pilot laboratory for virus production in Cascavel, PR, Brazil and by the end of that year was inoculating 100,000 *A. gemmatalis* larvae per day, employing 14 people. After processing 1,000 kg of dead larvae, viral yield was 65–72 ha-equivalent/kg. Coodetec subsequently built large laboratory facilities in 2004, consisting of two independent laboratories of 750 m^2 each: one for insect production and the other for virus production, with another facility (500 m^2) for virus storage, processing, and formulation. In the first laboratory, eggs are collected daily in adult oviposition rooms, and larvae are reared in separate rooms up to the 4th instar in 500-ml cardboard cups containing insect diet. Daily, 4–5% of the 4th instar larvae are transferred to plastic trays with diet and vermiculite to obtain pupae and maintain the insect colony. The remaining larvae (95–96%) are taken to the virus production laboratory where they are transferred from the 500-ml cups to plastic trays containing AgMNPV-treated diet. Seven days later, dead larvae are collected in plastic bags and stored at −4°C for further processing and formulation of the biopesticide. The laboratories implemented at Coodetec were implemented to employ 45 people to inoculate 800,000–1,000,000 larvae per day, resulting in a quantity of AgMNPV to treat 1.8–2.0 million ha/year. However, these laboratories discontinued their production as soybean pest control strategy changed in the last 7 years, drastically reducing the demand for the AgMNPV, which is discussed below.

16.4.1.4 Why Did the AgMNPV Program Experience a Setback in Brazil?

With the implementation of no-till agricultural systems in Brazil, soybean farmers had to apply herbicides as desiccants prior to soybean sowing. In this operation, growers began to mix broad-spectrum insecticides (such as pyrethroids) with the herbicides, "just to kill any insects that were present in the weeds" (Corrêa-Ferreira et al. 2010). At 15–20 days after soybean emergence, when growers apply postemergence herbicides, most also mixed pyrethroids, and in most cases, these two insecticide applications before and early in crop development were found to be detrimental to natural enemies (predators and parasitoids), thus disturbing the equilibrium in soybean systems. Other insects/organisms such as the soybean looper, white flies, *Spodoptera* spp., and mites, which were considered as secondary pests 7 years ago then became important pests (Bueno et al. 2007). Farmers, therefore, went into a treadmill, as broad-spectrum chemicals had to be applied against these "pests," and a highly specific biological product such as AgMNPV could no longer be used. Coodetec ceased production of the virus in the laboratory, but the developments regarding commercial AgMNPV production under laboratory conditions were proved to be viable and cost-competitive with chemical insecticides available on the market. Presently, this virus is used on about 300,000 ha yearly, compared to about two million hectares 7 years ago (Moscardi 2007). Lastly, with the introduction of soybean rust in Brazil, at least two applications of fungicides are made that may reduce the incidence of important natural entomopathogenic fungi such as *Nomuraea rileyi* (Sosa-Gómez et al. 2003) and Entomophthorales, which used to hold down populations of various caterpillar species before fungicide applications on the crop.

16.5 Factors Limiting Baculovirus Use

A successful program for use of a baculovirus depends upon a combination of factors (see Moscardi 1999 and references cited in pgs. 274–277), including selection of the most virulent isolate, application timing (as larvae may take a week or more to die), application technology, and plant substrate. However, solar radiation is the major factor affecting field persistence of baculoviruses. Viral activity can be completely lost in less than 24 h, but mean half-life generally has varied from 2–5 days. Ultraviolet radiation in region B (UV-B) (280–310 nm) inactivates baculoviruses. However, UV-A (320–400 nm) may also be critical in baculovirus deactivation. Many substances have been tested as sunscreening agents in formulations of these biological products, with many promoting protection to baculoviruses against UV radiation, such as fluorescent brighteners of the stilbene group. Besides protection against UV, the stilbenes also enhance viral activity (Shapiro 1995, Morales et al. 2001 and literature cited therein).

Baculoviruses, because of their high specificity, are most suited for use in agriculture, forestry, and fruit crop systems where there are no concurrent

important insect pests, as was the case of the application of AgMNPV against *A. gemmatalis* in soybean in Brazil (Moscardi 1999). Also, if an IPM program is not adopted by farmers, it is difficult to succeed in using a baculovirus, since the target insect must be monitored frequently (at least once per week) to time applications against the most susceptible larval instars (i.e., young ones). Farmers may find the need to sample their fields every week troublesome and may prefer the rapid killing by chemical insecticides. A critical issue in baculovirus use is the time necessary to kill the insect host (Moscardi 1999; Szewczyk et al. 2006, 2009; Souza et al. 2007; Erlandson 2008). Farmers may not initially be prepared to observe no obvious control results for 4–5 days following application of a baculovirus insecticide (Moscardi 2007). In Brazil, in the beginning of the AgMNPV program, farmers were not accustomed to wait long for *A. gemmatalis* larval mortality after virus application. Many would return to the fields within 2 or 3 days and apply chemical insecticides, not waiting for the virus to act on the larval populations. Because of this limitation, research has been directed at developing genetically modified baculoviruses with shorter times to kill their host larva.

16.6 Genetically Modified Baculoviruses to Control Insects

In the past, the practical application of baculoviruses as commercial insecticides was hampered by their relatively slow killing action and technical difficulties for *in vitro* commercial production. Due to the slow killing action, primary users (used to fast-killing chemical insecticides) regarded baculoviruses as ineffective. With advances in genetic-engineering technologies, many successes have been made in improving the timing of the killing action. Two broad strategies have been pursued in laboratories worldwide to achieve this goal: interference with host physiology and introduction of an insect-specific toxin (Bonning and Hammock 1996; Mishra 1998; Inceoglu et al. 2001).

The first strategy involves introducing genes coding for some insect hormones or enzymes into the baculovirus genome. Alternatively, the deletion of some non-essential baculovirus genes provides a beneficial effect for the speed of kill of a virus, as was found in the case of viral ecdysteroid UDP-glucosyltransferase (*egt*) gene. The product of this gene catalyzes the conjugation of sugar molecules to ecdysteroids (Tumilasci et al. 2003), thus preventing the ecdysteroid from crossing cellular membranes. Maeda (1989) was the first to introduce a diuretic hormone gene into *Bombyx mori* baculovirus genome to cause insects to lose water. Modified BmNPV killed larvae about 20% faster than wild-type BmNPV. Ma et al. (1998) expressed pheromone biosynthesis activating neuropeptide (PBAN) fused to the bombyxin signal sequence for secretion using AcMNPV. The recombinant baculovirus reduced survival time of *Trichoplusia ni* larvae by more than 20% in comparison to larvae infected with a control virus. Two other insect

hormone genes (eclosion hormone and prothoracicotropic hormone genes) were also studied as potential factors for modification of baculovirus; however, no significant improvement over wild-type virus was observed (Eldridge et al. 1991; O'Reilly et al. 1995). Another strategy for improving the timing of the killing action was based on control of the juvenile hormone, which in lepidopteran larvae regulates the onset of metamorphosis at the final molt. The juvenile hormone is regulated by juvenile hormone esterase which when overexpressed decreases concentration of the hormone. This, in turn, is a signal to stop feeding and to pupate. This elegant hypothesis for improvement of baculovirus action encountered many difficulties in practice but is being pursued to make it more efficient under natural conditions (Hammock et al. 1990; van Meer et al. 2000; Hinton and Hammock 2003; Inceoglu et al. 2001).

Another approach to reduce killing time was used by O'Reilly and Miller (1991), who deleted the baculovirus-encoded ecdysteroid glucosyltransferase gene. The product of the *egt* gene normally prevents larval molting during infection and indirectly increases feeding activity of infected caterpillars. The infection with recombinant virus resulted in 30% faster killing of larvae and significant reduction in food consumption. The egt enzyme is responsible for rendering the hormone ecdysone inactive. Inactivation of ecdysone results in prolongation of the larval stage and increased plant consumption. When larvae are infected with an *egt*-minus virus, molting proceeds normally, and consequently, the larvae eat less food. The *egt* gene is not essential for viral replication and can be replaced with an exogenous gene, e.g., with a toxin gene, which may further enhance the insecticidal activity of the recombinant virus (Popham et al. 1997; Sun et al. 2004).

Enhancins are baculovirus-encoded proteins that can increase the oral infectivity of a heterologous or homologous baculovirus. Their infection-enhancing effects are probably due to the degrading action on mucins and to the improved fusion of the virus to the midgut epithelium cells (Wang et al. 1994; Wang and Granados 1997). Enhancin genes have been expressed by recombinant AcMNPVs and subjected to dose-mortality studies (Hayakawa et al. 2000; Li et al. 2003). LD_{50} values were significantly lower for the recombinant virus in comparison to the wild-type virus (from 4.4- to 21-fold lower). Harrison and Bonning (2001) have constructed a recombinant AcMNPV producing three different proteases from the flesh fly *Sarcophaga peregrina*, which are known to degrade basement membrane proteins. One of the recombinants expressing cathepsin L under baculovirus promoter of *p6.9* gene generated a 51% faster speed of kill in comparison to the wild-type virus. Chitinases are enzymes that degrade chitin into low-molecular-weight oligosaccharides. Baculovirus chitinases are likely to be involved in the degradation of exoskeletons and gut linings of insects. A recombinant AcMNPV expressing the chitinase gene of *Manduca sexta* was constructed by Gopalakrishnan et al. (1993). When fourth instar *Spodoptera frugiperda* larvae were infected with the recombinant, their survival time was reduced by approximately 1 day in comparison to the wild-type AcMNPV.

Modification of the baculovirus genome by introduction of specific toxin genes has been much more widely exploited than methods based on interference with host physiology. Most reported research has focused on arthropod toxin genes isolated from mites, spiders, or scorpions (reviewed by Inceoglu et al. 2001; Kamita et al. 2005a, b). This line of research proved to be highly successful, but the reluctant attitude of policy makers in many countries toward genetically engineered products has hampered their introduction. The first reports on successful construction of baculovirus genome containing insect-specific toxin genes were published about 20 years ago (Carbonell et al. 1988; Tomalski and Miller 1991). The most promising insect-specific toxin gene used for construction of baculovirus recombinants is probably the gene coding for AaIT toxin originating from the scorpion *Androctonus australis*. The reported speed of kill by this baculovirus recombinant was increased up to about 40%, and the feeding damage was also reduced by about 40% (Inceoglu et al. 2001). The AaIT toxin gene was introduced into different baculovirus vectors including NPVs of *Bombyx mori* (Maeda et al. 1991), *Autographa californica* (Stewart et al. 1991), mint looper *Rachiplusia ou* (Harrison and Bonning 2000), cotton bollworm *Helicoverpa zea* (Treacey et al. 2000), and *Helicoverpa armigera* (Sun et al. 2004). Baculovirus expression of AaIT provides a continuous supply of freshly produced toxin; therefore, a low level of constant toxin production, even when driven by an early promoter, may be sufficient to elicit a paralytic response. In accordance with this hypothesis, Elazar et al. (2001) found that the concentration of AaIT in the hemolymph of paralyzed *Bombyx mori* is about 50 times lower when the toxin is delivered by a recombinant baculovirus in comparison to the dose delivered by direct injection of the same toxin. Toxin genes isolated from other scorpions, e.g., *Leiurus quinquestriatus hebraeus* (Chejanovsky et al. 1995; Gershburg et al. 1998; Froy et al. 2000), straw itch mite *Pyemotes tritici* (Burden et al. 2000), ants (Szolajska et al. 2004), or spiders *Diguetia canities* and *Tegenaria agrestis* (Hughes et al. 1997) and introduced into baculovirus genomes were highly active against lepidopteran larvae and are also under intensive study as potential biopesticides. Most of these toxins attack insect sodium channels, so their target is similar to chemical pesticides belonging to the pyrethroid group (Bloomquist 1996; Cestele and Catterall 2000). However, their specific site of action within sodium channels is different, so they may impart a synergistic effect when used in conjunction with baculovirus recombinants carrying toxin genes (McCutchen et al. 1997). Another promising approach for improvement of baculovirus insecticidal efficacy was suggested by Herrmann et al. (1995), who demonstrated that when excitatory and depressant toxins are simultaneously injected into insect larvae, they may exert a synergistic effect. Regev et al. (2003) have shown that in the case of a recombinant AcMNPV expressing toxin pairs (a combination of excitory and depressant scorpion toxins) used against *H. virescens*, *H. armigera*, and *Spodoptera littoralis* larvae, a cooperative insecticidal effect is observed. The recombinant producing excitory toxin LqhIT1 and depressant toxin LqhIT2 from *Leiurus quinquestriatus hebraeus* provided an improvement of 40% in effective time to paralysis when

compared to wild-type AcMNPV and an improvement of approximately 20% when compared to recombinants producing each toxin separately. Chang et al. (2003) have elaborated a novel and highly successful method for the improvement of recombinant baculoviruses; they generated a baculovirus that produced occlusion bodies incorporating Bt toxin. The recombinant baculovirus genome coded for native polyhedrin and a fusion protein in which polyhedrin is fused to the Bt toxin. The speed of action and pathogenicity of the recombinant were greatly enhanced compared to wild-type virus, thus yielding a biopesticide combining the positive properties of the virus and the bacterial toxin and minimizing the probability of evolution of insect resistance to these two killing factors.

Numerous studies have investigated the effectiveness of factors such as gene promoters and signal sequences in front of cloned genes on the efficiency of production and biological quality of expressed toxins. Historically, initial laboratory studies with recombinant baculoviruses were carried out by infecting caterpillars through ingestion of occlusion bodies or by injecting the budded virus into the hemocoel (O'Reilly et al. 1992). The infection by ingestion of occlusion bodies can be used for recombinants with healthy polyhedrin gene, so in the past, the toxin gene was usually introduced into the p10 locus, while the latter method was employed for recombinants with foreign genes in the polyhedrin locus. As an alternative to larval injections, the recombinant occlusion-negative viruses were packaged into polyhedra by cells infected with a second, occlusion-positive virus (e.g., wild-type virus) (Wood et al. 1993). A breakthrough in the construction of viral recombinants was the elaboration of the method of duplication of a viral promoter (Roy 1992). This procedure allowed for the expression of foreign genes under different promoters, e.g., under a basic protein gene promoter (Bonning et al. 1994) because none of the viral genes are lost. The level of recombinant gene expression in the baculovirus system is promoter-dependent, but factors other than the quantity of the product must also be taken into account. The argument for use of late or very late promoters in recombinant baculoviruses is the reduction of risk that a toxin gene could be expressed in nontarget insects because these promoters are not active in beneficial insects (McNitt et al. 1995). *Rachiplusia ou* MNPV (RoMNPV) expressing a gene coding for either scorpion *Androctonus australis* toxin (AaIT) or *Leiurus quinquestriatus hebraeus* toxin (LqhIT2) killed larvae of corn borer *Ostrinia nubilalis* most effectively when the gene was cloned behind a late p6.9 promoter. When p10 promoter was used, the level of polyhedra production was reduced in some cases, and virions were not occluded efficiently (Harrison and Bonning 2000). Recombinant AcMNPV expressing cathepsin L of the flesh fly through ie-1 promoter killed *H. virescens* larvae only slightly faster than wild-type AMNPV, but when the gene was expressed from the p6.9 promoter, the recombinant virus killed the host about 50% faster than did the wild-type baculovirus (Harrison and Bonning 2001). On the other hand, Tuan et al. (2005) showed that the early p-PCm promoter was superior to the very late p10 for controlling insect pests when LqhIT2 scorpion depressant toxin gene was introduced into AcMNPV genome, which may indicate higher susceptibility of earlier instars of these larvae to baculovirus infec-

tion. Sun et al. (2004) constructed a chimeric promoter by insertion of a p6.9 promoter downstream of the polyhedrin promoter and used this dual promoter for the expression of AaIT scorpion toxin gene in *egt* locus of HaSNPV. This HaSNPV-AaIT recombinant was found to be a much more effective biocontrol agent than the wild-type virus or *egt*-deleted virus.

Speed of action of genetically modified baculoviruses can be also enhanced by signal sequences in front of cloned genes. van Beek et al. (2003) constructed a series of AcMNPV recombinants expressing LqhIT2 scorpion toxin gene with different signal sequences, including signal sequences of AcMNPV GP64, cuticle protein II of *Drosophila melanogaster*, bombyxin of *B. mori*, dipteran chymotrypsin, and some scorpion toxins. Bombyxin signal sequence proved to be the most effective for enhancing insecticidal efficacy. Further searches for new promoters and for more effective signal sequences in transporting a toxin outside of the expressing cell are being carried out in many laboratories, and it is expected that many more natural and synthetic promoters and signal sequences will improve the speed of kill and safety of recombinant baculoviruses.

Biosafety of a biopesticide is an important problem, which requires special consideration. Biosafety can never be assured with absolute confidence, but a number of studies indicate that baculoviruses pose no hazard to animals other than their hosts. Though baculoviruses can enter mammalian cells, productive viral infection does not occur even at very high multiplicity of infection (Kost et al. 2005). Additionally, the foreign gene to be expressed after baculovirus infection must be placed under specific mammalian promoters; the expression from the baculoviral promoter has never been observed. Recombinant HaSNPV expressing AaIT scorpion toxin gene was not pathogenic to bees, birds, fish, and other vertebrates (Sun et al. 2002). Genetically modified AcMNPV did not affect the aquatic microbial community in any respect (Kreutzweiser et al. 2001). Natural enemies of larvae such as parasitoids and predators were not adversely affected by preying upon larvae infected with recombinant viruses (Li et al. 1999; Smith et al. 2000; Boughton et al. 2003). Also, it has not been proven thus far that the foreign gene can be transferred from donor recombinant baculovirus to another organism (Inceoglu et al. 2001, 2007). On the basis of these reports, it can be concluded that there is no evidence that recombinant baculoviruses pose greater threats to the animal world and the biosphere than the parental baculoviruses. However, in spite of this fact, field trials of genetically modified baculoviruses have instigated massive public protests, which put further trials on hold. The slow progress in application of genetically modified baculoviruses as pesticides may be, in part, due to the choice of “exotic” toxin genes used for modifications of the baculovirus genome. Taking into account the origin of these social conflicts, the choice of toxins used for this purpose should be reexamined, and baculoviruses should be modified with genes coding for more “natural” insect toxins, e.g., with genes coding for toxic polypeptides of parasitoid wasps occurring in regions infested by a particular pest.

16.7 Final Considerations and Further Prospects on Use of Baculoviruses as Biopesticides

Baculovirus insecticides have not met their full potential to control pest insects worldwide. In his review, Moscardi (1999) previewed the following: (1) The expansion of baculovirus use, in the following 5 years, i.e., up to 2004, would depend on new developments in the areas of recombinant baculoviruses and in the *in vitro* commercial production of these agents. The development of recombinant baculovirus was efficiently completed by researchers in several countries, but the *in vitro* commercial technology still lags behind today due to technical problems; (2) The use of baculoviruses would increase substantially in 10 years (i.e., up to 2009). However, this did not occur; (3) The AgMNPV program in Brazil could reach about four million hectares of soybean. This did not happen either. In reality, the use of the AgMNPV declined from two million hectares to about 300,000 ha over the past 7 years due to reasons discussed above (Sect. 16.4.1.4). In spite of this reduction in AgMNPV usage, this program can be considered an example regarding the viability of baculoviruses as insecticides. A current program for revival of the integrated pest management of soybean insect pests in Brazil will help to increase the use of AgMPV.

Despite the low use of viral insecticides worldwide (ca. 0.5%) as compared to biopesticides based on the bacterium *Bacillus thuringiensis*, total use of microbial insecticides worldwide is only about 2.0–2.5% of the total market of insecticides. Despite the low market influence of baculovirus insecticides, there are over 50 registered products in different countries, including the same product under different trade names. In the future, genetically modified baculoviruses will contribute to the expansion of baculovirus use worldwide, as these GMOs are considered safe through extensive research conducted over many years (Szewczyk et al. 2009). The most important issue for baculovirus use will be public perception regarding the benefits of baculovirus GMOs to control insects, including low impact on the environment. Also, regardless of whether a program is based on a wild-type or a genetically engineered baculovirus, global farmer education toward general use of biological pest control agents will be a key feature for expansion of baculovirus use worldwide. Unfortunately, pest control programs in most countries are directed toward the use of chemical insecticides, as in Brazil, where the official extension services have been "demolished" in 90% of the states over the past 10 years, leaving farmers to the control recommendations of professionals related to agrochemical companies. The use of baculoviruses as very specific bioinsecticides will depend on sound IPM programs, where integration of available techniques to control insects are used to reduce the number of chemical insecticide applications on a given crop and minimize the environmental impact of pest control. In systems where no IPM programs exist, there is little chance of success of use of a very specific baculovirus, especially in crop production systems where the one to be controlled with

a baculovirus occurs with other concurrent insect pests. Adoption of the IPM approach by farmers is important for use of baculovirus pesticides for successful sustainable agriculture.

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Chapter 17
Fungal Bioinoculants for Plant Disease Management

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Abstract Plant diseases are among the major constraints in the production of food crops and inflict significant losses to global agriculture. Pesticides are widely used to control plant diseases but their application is costly and, in some cases, may bring more disadvantages than benefits. Use of bioinoculants to control plant diseases is an economically viable and ecologically sustainable method of disease management. A large number of bioinoculants is available; among them, bioinoculant fungi constitute the majority and are widely used in different cropping systems. Important bioinoculants that directly parasitize plant pathogens include *Trichoderma* spp., *Paecelomyces lilacinus,* and *Pochonia chlamydosporia*. Plant growth-promoting fungi such as *Aspergillus* spp. and *Penicillium* spp. may also suppress plant pathogens. In general, bioinoculants are effective against seed- and soil-borne fungi and nematodes. However, an important limitation in their commercial use in crop protection is nonavailability of efficient immobilizing systems for delivery and survival of bioinoculants. This chapter describes important bioinoculants, their effects, and their mechanisms of action against plant diseases caused by fungi, bacteria, and nematodes. State-of-the-art technology available for the production of commercial formulation of bioinoculants, along with important lacuna, is also discussed.

17.1 Introduction

Plant diseases are a common component of natural systems and are among many ecological factors that keep plant and animal populations in balance. When a plant suffers from an infection, its normal development and functioning are affected and

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_17,

it becomes diseased. Kuhn, in 1858 (Wilhelm and Tietz 1978), was probably the first to scientifically define plant disease as "abnormal changes in physiological processes which disturb the normal activity of the organs." Ward (1896) defined disease as a condition in which the function of the organism is improperly discharged or, in other words, it is a state which is physiologically abnormal and threatens the life of the being or organ. The British Mycological Society defined disease as a harmful deviation from the normal functioning process (Wallace et al. 1950). Plant disease can also be defined as "a physiological disorder or structural abnormality that is harmful to the plant or to any of its parts or products that reduces the economic value" (Stalkman and Harrar 1957). According to Horsfall and Cowling (1977), disease is a malfunctioning process that is caused by continuous irritation which may result in some suffering, and this produces symptoms. More scientifically, "disease is any malfunctioning of host cells and tissues that results from continuous irritation by a pathogenic agent or environmental factor and leads to development of symptoms" (Agrios 2005).

Crop plants are known to be affected by over one hundred diseases (Agrios 2005). However, only a few, usually a single pathogen, at a given time can multiply to an extent to cause the disease. Diseases of crop plants are among the most important constraints in the production of adequate quantities of food. Approximately half of the world`s total agricultural production is lost due to various pests and diseases at planting and postplanting stages (Khan 2008). The incidence of crop losses due to disease is much lower in developed countries because of awareness among farmers for disease management. In developing countries, greater yield losses occur due to plant diseases because of unplanned agricultural practices such as use of marginal lands, low agricultural inputs, and lesser concerns by farmers toward plant disease management. On average, losses inflicted by weeds, plant diseases, and insect pests upon agricultural crops have been estimated as 33, 26, and 22%, respectively (Khan 2008). According to another estimate, plant diseases, weeds, and insects contribute to a 14.1, 10.2, and 12.2%, respectively, decline in crop production (FAOSTAT 2003; Agrios 2005; Table 17.1). Among different kinds of pathogens, the greatest losses are inflicted by fungi (42%) followed by bacteria (27%), viruses (18%), and nematodes (13%) (Khan and Jairajpuri 2010; Fig. 17.1).

Table 17.1 Estimated annual crop losses caused by pests and diseases worldwide[a]

Practice	Losses (US $)
Attainable cop production (2002 prices)	$1.5 trillion
Actual crop production (−36.5%)	$950 billion
Production without crop protection	$455 billion
Losses prevented by crop protection	$415 billion
Actual annual losses to world crop production	$550 billion
Losses caused by disease only (14.1%)	$220 billion

[a]FAOSTAT (2003); Agrios (2005)

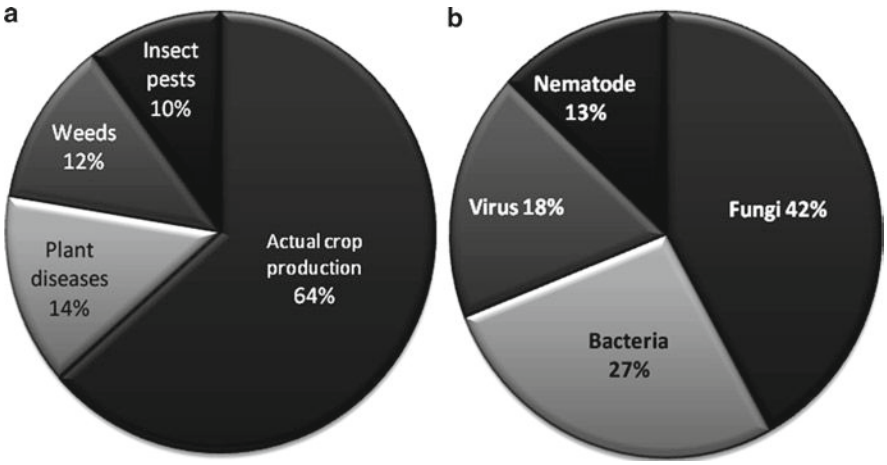

Fig. 17.1 Actual crop production and annual crop losses due to plant diseases, insect pests, and weeds (**a**) and breakdown of crop losses caused by fungi, bacteria, viruses, and nematodes (**b**)

17.1.1 Management of Plant Diseases

Continual increases in global human population have put twofold pressure on agriculture. Precious agricultural lands are being diverted from crop production to urbanization and industrialization. As a result, the net area under crop production is shrinking, whereas demand for food products continues to increase at an alarming pace. According to one estimate, the present global land area under crop production would produce much greater quantities of food than present requirements if pest- and disease-free crops were grown (Khan and Jairajpuri 2010). Hence, the primary requirement to meet food requirements of both present and future populations is to integrate plant protection techniques into crop production systems. Numerous methods of pest and disease management are available including chemical, cultural, physical, and biological, which are used according to the crop, pathogen, availability of material, and demand of the situation.

Pest control methods involving chemical pesticides is one of the most effective and reliable means of disease management; however, in an environmentally conscious world, the use of pesticides is under criticism because of several real and perceived ill effects. Age-old cultural practices like crop rotation, mixed cropping, green manuring, etc. to combat plant diseases are slow in action and are of no benefit during epidemic situations. The pace of development and durability of resistant/tolerant crop cultivars has been slow and unreliable in spite of tremendous advancements in plant genetic engineering. Considering these limitations, there has been a growing emphasis on the development of novel management practices that alone or in integration with other practices result in a good degree of reduction in pathogen inocula and disease severity coupled with sustainability in the production system, cost-effectiveness, and eco-friendliness. Biological control is an important approach

in this direction. The most obvious and environment-friendly alternative to pesticides is to use naturally occurring beneficial bioinoculants to manage pests and diseases.

17.1.1.1 Biological Control

Consensus is developing that chemical-based farming is nonsustainable; as a result, ecological approaches are being researched more intensively. The most obvious environment-friendly alternative to pesticide application for managing agriculturally important diseases is the use of biological approaches. Biological control is based on the phenomenon that every living entity has an adversary in nature to keep its population in check (Khan 2005). In 1965, Garrett defined biological control as "any condition under which, or practice, whereby, survival and activity of a pathogen is reduced through the agency of any other living organism (except man himself) with the result that there is a reduction in the incidence of disease caused by the pathogen." Baker and Cook (1974) defined biological control as the "reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonists, or by mass introduction of one or more antagonists." In 1983, they revised the definition to "the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by one or more organisms other than man."

Biological control can be achieved either by introducing bioinoculants (biocontrol agents) directly into a natural ecosystem or by adopting cultural practices that stimulate survival, establishment, and multiplication of the bioincoulants. Hence, more scientifically, biological control of pests and diseases can be defined as reduction in disease severity, crop damage, population or virulence of the pest or pathogen in its active or dormant state by the activity of microorganisms that occur naturally through altering cultural practices which favors survival and multiplication of the microorganisms or by introducing bioinoculants.

In 1874, Roberts demonstrated the first evidence of antagonistic action of microorganisms in liquid cultures between *Penicillium glaucum* and a bacterium and introduced the term "antagonism" (Baker 1987). Since then, a great deal of data has been generated to demonstrate that biological control is a realistic proposition for disease management. The first attempt to control a plant disease with microorganism introduced to soil was by Hartley in 1921 where introduction of isolates of saprophytic fungi and one bacterium resulted in significant reduction in severity of damping-off of pine seedlings caused by *Pythium debaryanum* (Baker 1987).

Bioinoculant Fungi and Mechanisms of Action

Bioinoculants or biocontrol agents are the microorganisms that induce stimulatory effects on plant growth and/or suppressive effects on pests or pathogens through

a variety of mechanisms when applied in an ecosystem. A large number of bioinoculants have been investigated to harness their beneficial effects on crop productivity. Bioinoculants are primarily fungal and bacterial in origin. Bioinoculant fungi basically work through parasitism (Papavizas 1985; Stirling 1993) against plant pathogenic fungi and nematodes (Khan 2005). The important genera of biocontrol fungi that have been tested against plant pathogenic fungi and nematodes include *Trichoderma*, *Aspergillus, Chaetomium, Penicillium, Neurospora, Fusarium* (saprophytic), *Rhizoctonia, Dactylella, Arthrobotrys, Catenaria, Paecilomyces, Pochonia,* and *Glomus*. Other kinds of biocontrol agents such as plant growth-promoting organisms have also been evaluated for disease management (Papavizas 1985; Nair and Burke 1988). A number of fungi such as *Aspergillus* spp., *Penicillium* spp., and *Trichoderma* spp. are active phosphate-solubilizing microorganisms (PSM), which also suppress plant pathogens. Application of PSM can control soil-borne pathogens such as *Fusarium oxysporum, Macrophomina phaseolina, Pythium aphanidermatum, Rhizoctonia solani, Sclerotinia sclerotiorum*, and *Meloidogyne incognita* (Sen 2000; Khan and Anwer 2007, 2008; Khan et al. 2009).

Bioinoculants suppress plant pathogens by direct parasitism, lysis, competition for food, direct antibiosis or indirect antibiosis through production of volatile substances, viz., ethylene, hydrogen cyanide, alcohols, monoterpenes, and aldehydes (Juan et al. 2005). Activity of bioinoculants mainly depends on the physicochemical environmental conditions to which they are subjected. These mechanisms are complex, and what has been defined as biocontrol is the final result of varied mechanisms acting antagonistically to achieve disease control. Some important mechanisms in disease suppression by bioinoculants are discussed below.

Fungistatic

An effective antagonist is usually able to survive in the presence of metabolites produced by other microorganisms and plants, and multiply under extreme competitive conditions. *Aspergillus* spp., *Penicillium* spp., and *Trichoderma* spp. were found to be most resistant to herbicides, fungicides, pesticides, and many toxic heavy metals at minimum inhibitory concentrations (MIC) of 125–850 μg/ml (Baytak et al. 2005; Yuh-Shan 2005; Ahmad et al. 2006; Braud et al. 2006). Dose–response relationships of fungicide resistance in agar growth tests were examined with *Aspergillus niger, A. nidulans*, and *Penicillium expansum* to pentachloronitrobenzene (PCNB), 3-phenylindole, benomyl, or thiabendazole, and resistance was measured at high concentrations of these chemicals (van Tuyl 1977). When *A. niger* was included with Foltaf SOW (Captafol 80%) for the treatment of pigeonpea wilt, the disease was more effectively controlled than when the fungicides were used alone (Bhatnagar 1995).

Trichoderma strains grow rapidly when inoculated in soil because they are naturally resistant to many toxic compounds such as DDT and phenolic compounds (Chet et al. 1997). *Trichoderma* strains are efficient in controlling several phytopathogens such as *R. solani*, *P. ultimum* and *S. rolfsii* when alternated with methyl

bromide, benomyl, captan, or other chemicals due to the presence of the ABC transport system (Vyas and Vyas 1995; Harman et al. 2004). When *Trichoderma harzianum* was included with Blue Copper-50 for the treatment of pigeon-pea wilt, the disease was more effectively controlled than when the fungicides were used alone (Bhatnagar 1995).

Competition for Nutrients

Starvation or shortage of nutrients is one of the most common causes of death of microorganisms (Chet et al. 1997). Competition resulting in limiting the nutrient supply to fungal phytopathogens results in their biological control (Chet et al. 1997). For instance, in most filamentous fungi, iron (Fe) uptake is essential for viability (Eisendle et al. 2004), and under Fe-deficient condition, most fungi excrete low-molecular-weight ferric iron-specific chelators termed siderophores to mobilize environmental Fe (Eisendle et al. 2004). Siderophores play a considerable role in biocontrol of soil-borne plant pathogens (Leeman et al. 1996) and as a supplier of Fe nutrition to crop plants (Jadhav et al. 1994). Since plant pathogens may not have the cognate ferri–siderophore receptor for uptake of the Fe–siderophore complex, they are prevented from proliferating in the immediate vicinity because of Fe deficiency (O'Sullivan and O'Gara 1992). Hence, siderophore-producing bioinoculants can confer a competitive advantage to interactions in the rhizosphere (Raijmakers et al. 1995). One of the most sensitive stages for nutrient competition in the life cycle of *Fusarium* is chlamydospore germination (Scher and Baker 1982). In soil, the chlamydospores of *F. oxysporum* require adequate nutrition to maintain a germination rate of 20–30%. Germination may decrease due to sharing of nutrients with other microorganisms. Root exudates are a major source of nutrients in soil. Thus, colonization in the rhizosphere by an antagonist might reduce infection by *Fusarium*-like pathotypes (Cook and Baker 1983). *Aspergillus niger* AN27, a potential biocontrol agent, produced both hydroxamate and catecholate groups of siderophores (Sen 1997; Mondal and Sen 1999).

Trichoderma has a superior capacity to mobilize and take up soil nutrients compared to other microorganisms. The efficient use of available nutrients is based on the ability of *Trichoderma* to obtain ATP from the metabolism of different sugars, such as those derived from polymers widespread in fungal environments, for example cellulose, glucan, and chitin among others, all rendering glucose (Chet et al. 1997). High-affinity glucose transporter, Gtt 1, has been isolated from *T. harzianum* CECT 2413. Role of this transport system is yet to be discovered properly, but its efficiency is considered to be crucial in microbial competitions (Delgado-Jarana et al. 2003). The strain CECT 2413 was present in nutrient-poor environments and relied on extracellular hydrolases for survival. The Gtt 1 is only expressed at very low glucose concentrations, that is, when sugar transport is expected to be limiting in nutrient competition (Delgado-Jarana et al. 2003).

By the same mechanism, soil composition influences the biocontrol effectiveness of *Pythium* by *Trichoderma* (i.e., according to Fe availability). Some *Trichoderma* strains produce highly efficient siderophores that chelate Fe and stop

the growth of other fungi (Chet and Inbar 1994). In addition, *T. harzianum* T35 controls *F. oxysporum* by competing for both rhizosphere colonization sites and nutrients, with biocontrol becoming more effective as the nutrient concentration decreases (Tjamos et al. 1992). Competition for carbon has also been involved in the occurrence of antagonism expressed by different strains of *Trichoderma* spp. against plant pathogens, particularly *F. oxysporum* (Sivan and Chet 1989). The advantage of using *Trichoderma* to control *Botrytis cinerea* is the coordination of several mechanisms, the most important being nutrient competition, since *Botrytis cinerea* is particularly sensitive to low nutrient levels (Latorre et al. 2001).

Antibiosis

Antibiosis is the phenomenon of suppression of one organism by another due to release of toxic substances/metabolites into the environment. Antibiosis is important in determining the competitive saprophytic and necrotrophic ability of antagonists. The bioinoculant fungi may suppress plant parasitic nematodes through antibiosis and by stimulating host defense. Low-molecular-weight compounds and antibiotics (both volatile and nonvolatile) produced by *Trichoderma* species and *Aspergillus* spp. impede colonization of harmful microorganisms including nematodes in the root zone (Eapen and Venugopal 1995). Harzianic acid, alamethicins, tricholin, peptaibols, 6-pentyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid, oxalic acid, and enzymes are some of the chemicals possessing antibiotic properties produced by *Trichoderma* and *Aspergillus* species (Mankau 1969a, b; Benitez et al. 2004; El-Hasan et al. 2007).

Aspergillus spp. and *Trichoderma* spp. are well known for producing antifungal and antibacterial agents (Buchi et al. 1983; Fujimoto et al. 1993). An antifungal butenolide, harzianolide has been isolated from *Trichoderma harzianum* (Claydon et al. 1991). Most *Trichoderma* strains produce volatile and nonvolatile toxic metabolites that impede colonization by antagonized microorganisms; among these metabolites, the production of harzianic acid, alamethicins, tricholin, peptaibols, 6-pentyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid, and others have been described (Vey et al. 2001). In some cases, antibiotic production correlates with biocontrol ability, and purified antibiotics mimic the effect of the entire agent. Volatile substances from *Trichoderma* spp. inhibited mycelial growth of *Macrophomina phaseolina* by 22–51% (Angappan 1992). The volatile antibiotics of *T. harzianum* and *T. atroviride* significantly decreased growth of canker fungal pathogens of poplar, *Cytospora chrysosperma* and *Dothiorella gregaria*, but nonvolatile metabolites in the culture filtrate of *Trichoderma* spp. inhibited the linear growth of pathogens (Deshmukh and Pant 1992; Pandey 1988). There are also examples of antibiotic-overproducing strains such as gliovirin-overproducing mutants of *T. virens*, which provide controls similar to that of the wild type and of gliovirin-deficient mutants, which failed to protect cotton seedlings from *Phythium ultimum*, whereas the parental strain did (Chet et al. 1997). *Trichoderma* spp. are reported to produce carbon monoxide, ammonia (Dennis and Webster 1971b), carbonyl compounds, and acetaldehyde (Robinson and Park 1966), which may enhance antagonistic activity in soil.

Aspergillus niger, *Trichoderma* spp., and *Penicillium* spp. that parasitize eggs prefer eggs which are deposited in cyst or a gelatinous matrix. The oviposition nature of *Heterodera* spp. and *Meloidogyne* spp. makes them more vulnerable to attack by these fungi. As soon as the fungi identify a cyst or an egg mass, they rapidly grow and colonize those eggs where larval formation is not complete. However, when larva is formed, the egg becomes less vulnerable. It has been suggested that this differential vulnerability of egg and larval stage is due to chitinolytic activity of these fungi. Chitin is a major constituent of the egg shell, which is lacking in the larval cuticle.

The fungus *P. chlamydosporia* (i.e., *Verticillium chlamydosporium*) produces nematicidal metabolites. The culture filtrate of *P. chlamydosporia* in yeast extract medium showed pronounced nematicidal and nematostatic effects. A dilution of 1:1 culture filtrate caused 100% mortality of *G. rostochiensis, G. pallida*, and *Panagrellus redivivus* (Saifullah 1996c). The actively growing mycelium of *P. chlamydosporia* infects eggs and females of nematodes (Morgan-Jones et al. 1983). Egg hatching in the presence of the fungus was inhibited probably due to the effect of toxins secreted by the fungus (Meyer et al. 1990) or disintegration of the eggshell's vitelline layer and also partial dissolution of the chitin and lipid layers due to activity of exoenzymes (Lopez-Llorea and Duncan 1988; Saifullah and Thomas 1997; Stirling 1991). Serine proteases have been identified in *P. chlamydosporia* (Segers et al. 1994). These extracellular enzymes are synthesized in the presence of nematode eggs and repressed by glucose (Segers et al. 1999). In a chemical investigation of one fungal strain of *P. chlamydosporia*, YMF 1.00613, isolated from root knots of tobacco infected by *M. incognita,* four aurovertin-type metabolites were isolated and identified, including a new compound, aurovertin I (A1), and three known metabolites, aurovertins E, F, and D (A2–A4). The results suggest that the aurovertin-type metabolites produced by *P. chlamydosporia* might be one of the pathogenic factors involved in the suppression of nematode *M. incognita* (Niu et al. 2010).

Paecilomyces lilacinus is an effective parasite of nematode eggs and adults (Jatala et al. 1979) and its mode of action involves recognition phenomena (e.g., chemotaxis and adhesion), signaling and differentiation, and penetration of the nematode cuticle/eggshell using mechanical as well as enzymatic (protease and chitinase) means (Lopez-llorca et al. 2008).

Mycoparasitism

Mycoparasitism involves direct parasitism of one fungus by another and involves recognition, attack, and subsequent penetration and killing of the host fungus (Harman et al. 2004). In a necrotrophic association, there is direct contact between two fungi, and a nutrient exchange channel is established between them. Typical examples are the association of *Arthrobotrys oligospora* with *R. solani* (Persson et al. 1985), *Trichoderma hamatum* with species of *Phythium,* and *Rhizoctonia* with *Sclerotium* (Bruckner and Pryzybylski 1984).

Observations using scanning electron microscopy revealed that *A. niger* coiled around the pathogen hyphae and penetrated within. Presence of *A. niger* hyphae

inside pathogen hyphae has been confirmed using fluorescent microscopy repeatedly in *F. oxysporum* f.sp. *melonis* and *ciceris*, and other pathogens (Sen et al. 1997; Sharma and Sen 1991a, b). Further studies have revealed that *A. niger* could kill *Macrophomina phaseolina,* several species of *Pythium, Rhizoctonia solani, Sclerotinia sclerotiorum* (Sen et al. 1995), and *Sclerotium rolfsii* (Palakshappa et al. 1989). The dead hyphae of the pathogens were eventually invaded. These observations confirm that *A. niger* is a contact and invasive necrotroph (Mondal and Sen 1999).

Trichoderma spp. may detect a host fungus before contact and grow toward it. Such remote sensing is partly due to the sequential expression of cell wall-degrading enzymes, mostly chitinases, glucanases, and proteases (Harman et al. 2004). *Trichoderma* attaches to the pathogen with cell wall carbohydrates that bind to pathogen lectins. Once *Trichoderma* is attached, it coils around the pathogen and forms the appressoria. Production of cell wall-degrading enzymes and peptaibols (Howell 2003) follows, which facilitates both the entry of *Trichoderma* hypha into the lumen of the parasitized fungus and the assimilation of the cell-wall content. *Trichoderma* spp. reacts vigorously with hyphae of the *Fusarium* species. The hyphae of *Trichoderma* spp. when near a pathogen induce morphological deformities in the host hyphae. Many times bursting of hyphae and vacuolation have frequently been observed (Komatsu 1968; Gao et al. 2001). In addition, granulation, coagulation, disintegration, and finally lysis of the pathogen occurs (Lim and Teh 1990; Elad et al. 1983; Nigam et al. 1997; Gao et al. 2001). In vitro studies have revealed greatly suppressed synthesis of endochitinase, chitobiosidase, *n*-acetyl-b-glucosidase, and glucan 1, 3-β-glucosidase, and combinations thereof, during spore germination and germ tube elongation in *Trichoderma* spp. (Lorito et al. 1993; Di Pietro et al. 1993; Lorito et al. 1994a, b).

Stimulation of Host Defense Response

Association of *Trichoderma* spp., *Aspergillus* spp., *Penicillium* spp., and other phosphate-solubilizing fungal antagonists also stimulates plant defensive mechanisms (Howell et al. 2000; Hanson and Howell 2004). An elicitor of plant disease resistance, pectinase, was produced by *A. niger*, which elicited disease resistance in cucumber and tomato seedlings (Bai et al. 2004). Cervone et al. (1987) showed that the active endo-polygalacturonase (EPG) of *A. niger* formed oligosaccharides from pectin, which were capable of eliciting resistance response in *Vigna unguiculata*.

Species or strains of *Trichoderma* amended to the rhizosphere may also protect plants against aerial infections including those of viral, bacterial, fungal, and nematode pathogens, due to induction of resistance mechanisms similar to the hypersensitive response (HR), systemic acquired resistance (SAR), and induced systemic resistance (ISR) in plants (Harman et al. 2004). At the molecular level, resistance results in an increase in concentration of metabolites and enzymes related to defensive mechanisms, such as production of the enzymes phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), which are involved in the biosynthesis of phytoalexins (HR response), chitinases, and glucanases. These enzymes comprise pathogenesis-related proteins (SAR response) and enzymes involved in response to

oxidative stress. *Trichoderma* metabolites may act as elicitors of plant resistance or induce the expression in transgenic plants of genes whose products act as elicitors. The metabolites may also be instrumental in the synthesis of phytoalexins, PR proteins, and other compounds that may impart greater resistance against several plant pathogens, including fungi, bacteria, and nematodes (Elad et al. 2000; Howell et al. 2000; Dana et al. 2001; Hanson and Howell 2004), as well as resistance to stressful abiotic conditions (Harman et al. 2004). An ethylene-inducing xylanase (EIX) produced by *T. viride* (Dean and Anderson 1991) elicited the production of the phytoalexin resveratrol in grapevine cells (Calderon et al. 1993). Hanson and Howell (2004) reported that culture filtrates from effective biocontrol strains of *T. virens* stimulated significantly greater terpenoid levels in cotton, and the elicitors were most likely proteins or glycoproteins. *T. harzianum* also induced resistance in bean and cucumber (Koike et al. 2001).

Fungal Diseases and Their Management by Bioinoculants

Fungi are eukaryotes and constitute a group of plant pathogens that incite the most economically significant diseases of agricultural crops. Fungi infect all types of crops including cereals, vegetables, legumes, and ornamentals and cause specific symptoms (Fig. 17.2). Important diseases caused by fungi are rusts (*Puccinia* spp., *Hemileia* spp.), smuts (*Ustilago* spp., *Tilletia* spp.), seed-rot (*Pythium* spp.),

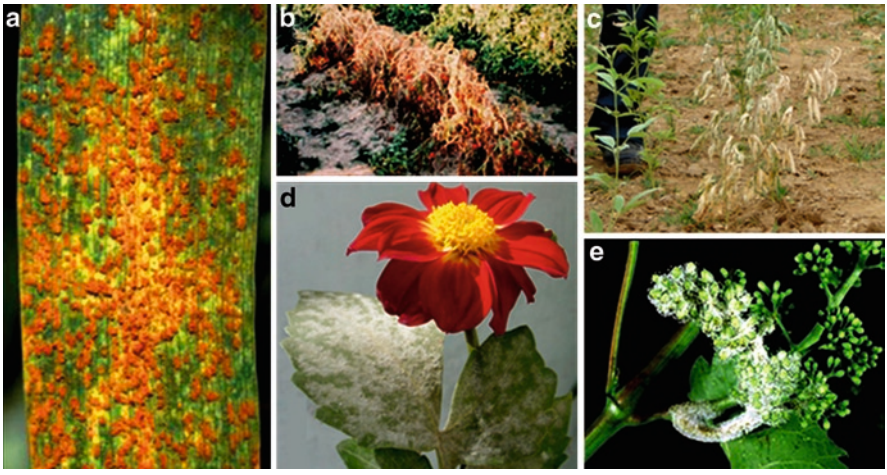

Fig. 17.2 Symptoms of some common plant diseases caused by fungi. (**a**) Leaf rust of wheat caused by *Puccinia recondite* f. sp. *tritici*, (**b**) Fusarial wilt of tomato caused by *Fusarium oxysporum* f.sp. *lycopersici*, (**c**) Fusarial wilt of pigeon pea caused by *Fusarium udum*, (**d**) Powdery mildew of dahlia caused by *Erysiphe cichoracearum*, (**e**) Downy mildew of grapes caused by *Plasmopara viticola*. (Courtesy photo: (**a**) http://www.ars.usda.gov/.../leaf%20rust%20poster.jpg; (**b**) http://www.mobot.org/.../images/Pests/Pest182.jpg; (**e**) http://www.plantmanagementnetwork.org/.../image/1sm.jpg)

damping-off (*Pythium* spp.), root rot (*Rhizoctonia* spp.), wilt (*Fusarium* spp.), blight (*Phytophthora* spp.), powdery mildew (*Erysiphe* spp., *Shaerotheca* spp.), and downy mildew (*Plasmopara* spp., *Peronospora* spp.), which attack crops under a varied range of agroclimatic conditions (Agrios 2005). Generally, moderately cooler climates with higher relative humidity are favorable for pathogenesis of fungi. Numerous studies have been conducted to test the effect of bioinoculants, and on several occasions, their application has proved quite effective in controlling fungal-induced plant diseases. The effects of the bioinoculants have been tested under in vitro, pot, and field conditions.

In Vitro

The antagonistic potential of *Trichoderma* spp. against plant pathogenic fungi has been widely explored. Bell et al. (1982) demonstrated in vitro antagonism of *Trichoderma* species against fungal pathogens. Cell-free culture filtrate of *T. virens* proved inhibitory to *Pythium ultimum* (Howell and Stipanovic 1983). *T. harzianum* strain C184 was tested in vitro for its antagonism against *Cylindrocladium pteridis*, which causes root necrosis in banana and plantain, and *Fusarium solani, F. oxysporum*, and *Aspergillus* sp., which are secondary colonizers of the root system of these crops (Ngueko 2002). *T. viride* and *T. harzianum* were screened for their antagonistic ability against the rice sheath blight pathogen, *Rhizoctonia solani,* and their culture filtrate inhibited the growth of *R. solani* (Krishnamurthy et al. 1999; Xu and Qin 2000).

Among five species of *Trichoderma*, *T. harzianum* and *T. viride* greatly suppressed the growth of *Macrophomina phaseolina* in a dual culture test (Khan and Gupta 1998). In a similar study, *T. virens* strongly antagonized *P. aphanidermatum,* the pathogen responsible for tomato damping-off disease. In fungal growth tests, the isolates *T. harzianum* 1, *T. harzianum* 2, *T. viride* 1, *T. viride* 2 and *T. viride* 3 inhibited growth of the *Helminthosporium* (*Bipolaris*) spp. by 79, 69, 84, 83 and 74%, respectively (Jegathambigai et al. 2009). *T. harzianum* was found antagonistic to *Rhizoctonia solani* and *Verticillium dahliae* at 15 and 25°C, respectively, and in vitro inhibited the development of *R. solani* and *V. dahliae* at both temperatures (Santamarina and Rosello 2006). Chaudhary and Prajapati (2004) reported antagonism of *T. harzianum* and *T. virens* against *F. udum.* The antagonists reduced colony growth of *F. udum* through saprophytic competition. *T. harzianum* showed maximal growth in a dual culture test and effectively inhibited the growth of *Macrophomina phaseolina* (65%) (Malathi and Doraisamy 2004). Similar effects of *T. harzianum* have also been reported on *S. rolfsii* (Prasad et al. 2003) and *F. udum* (Singh et al. 2002). In a dual culture test, *T. harzianum* caused severe vacoulation, shrinkage, and coagulation of the cytoplasm of pathogen hyphae.

In an in vitro study, *T. viride* inhibited the radial growth of *Aspergillus flavus* (51%), *A. fumigatus* (52%), *Fusarium* sp. (64%), and *Penicillium* sp. (54%) in dual culture (Rajendiran et al. 2010). *T. hamatum*, *T. pseudokoningii*, and *T. virens* inhibited *Phytophthora cinnamomi*, the causal organism of root rot of

chestnut, by mycoparasitism with evidence of parallel growth and coiling, and overgrowth, preventing further pathogen growth (Chambers and Scott 1995). Kucuk and Kivanc (2008) reported in vitro mycoparasitism of *Gibberella zeae* and *Aspergillus ustus* by *T. harzianum* strains. In another study, *Trichoderma* isolates were evaluated by the dual culture method, where competition by substrate, mycoparasitism, and antibiosis were observed. The *Trichoderma* spp. isolates inhibited the radial growth of *R. solani* between 60 and 98% (Martinez 2008).

Pot Culture

Species of *Trichoderma* provided protection to seeds during germination against seed rot fungi in pot culture (Elad and Chet 1987). In an in vitro experiment, Elad and Chet (1987) demonstrated that application of spores of *Penicillium oxalicum* on seeds, seedling roots, corms, bulbs, and tubers provided protection against *Pythium ultimum* (Elad and Chet 1987). Significantly lengthy protection from *Penicillium expansum* infection (up to 2 months) was obtained when intact apples were dipped for 30 s in formulated *T. harzianum* conidia before being inoculated by *P. expansum, as* compared to untreated fruits (Benitez et al. 2004).

Muskmelon seeds were soaked overnight in *Aspergillus niger* AN 27 (Kalisena SD) spore suspension and grown in sand for 6 days. The roots of seedlings (with fully opened cotyledonary leaves) were washed thoroughly in water to remove *A. niger* spores. The seeds were suspended in *F. oxysporum meloni* (aqueous) spore suspension. These Muskmelon seedlings raised from the *A. niger*-treated seeds showed 56% resistance to *F. oxysporum melonis* without physical presence of *A. niger* in the root zone. These seedlings were 58, 26, and 2% higher in peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase activity, respectively, over controls (Radhakrishna and Sen 1986; Angappan et al. 1996). The lignin content was also higher in the tissues of treated plants and resulted in the induced resistance (Kumar and Sen 1998).

Application of *Trichoderma* spp. has been found effective in pot conditions against a large number of fungi such as *Fusarium* spp. (Khan 2005), *Rhizoctonia* spp. (Olson and Benson 2007), *Macrophomina phaseolina* (Khan and Gupta 1998), *Pythium* spp. (Pill et al. 2009), *Phytophthora* spp. (Hanada et al. 2009), *Botrytis* spp., and other pathogenic fungi (Olson and Benson 2007). Greenhouse experiments showed that plant growth media based on grape marc compost (compost peat 1:1, v/v) amended with *T. asperellum* T34 suppressed Fusarium wilt of carnation (Sant et al. 2010). In another study, *T. koningii* (TNAU) was used to control chickpea blight caused by *Colletotrichum dematium* with seed treatment (10^8 cfu/ml) (Rao and Narayana 2010). In a greenhouse experiment, *Trichoderma* spp. isolates significantly controlled sheath blight of rice caused by *R. solani* (Martinez 2008) and Fusarium rot of bean caused by *Fusarium solani* (using a combination of *T. harzianum* and *T. asperellum*) (Ibrahimov et al. 2009). *T. asperellum* strain T34 also suppressed *Fusarium* wilt of carnation better than standard chemicals (Sant et al. 2010).

Field Conditions

In a field study, seed treatment with *T. harzianum* decreased incidence and severity of Fusarium wilt in chickpea by 30 and 60%, respectively (Khan et al. 2004). In another trial, the same antagonist provided the highest control of *F. oxysporum* f. sp. *ciceris*, which causes wilt in chickpea under field conditions (Singh et al. 2003). *T. harzianum* had superior antagonistic efficiency against ten isolates of *F. oxysporum* f. sp. *ciceri* compared to *T. viride* (Gurha 2001). Prasad et al. (2002) evaluated *T. harzianum* PDBCTH 10 and *T. viride* PDBCTV against natural incidence of chickpea wilt. The wilt incidence was highest (12 and 16%) in control plots, and in plots treated with *T. harzianum*, only 4 and 5.1% wilt incidence was observed at 60 and 90 days, respectively. Upadhyay and Mukhopadhyay (1986) demonstrated the suppression of *Sclerotium* root rot of sugar beet by application of *T. harzianum* in field soil. Singh and Singh (2004) reported that *T. harzianum* controlled *S. rolfsii*, the incidence of collar rot in mint by 67–100%. Khan and Akram (2000) observed a significant decrease in wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici* by soil application of *T. virens.* In another trial, soil application of *T. koningi, T. hamatum*, and *T. virens* controlled tomato wilt caused by *F. oxysporum* f. sp. *lycopersici* (Cipriano et al. 1989). Khan and Gupta (1998) reported superior control of root rot of eggplant caused by *Macrophomina phaseolina* following soil application of *T. harzianum* and *T. viride* in comparison to *T. koningi.* Satisfactory control of tomato damping-off has been reported by seed treatment with *T. virens* (De and Mukhopadhyay 1994).

Seed treatment with *T. harzianum* or *P. lilacinus* controlled wilt of tomato (Shahida and Gaffar 1991). Seed treatment with *T. harzianum* also checked root rot of chickpea caused by *R. solani*, and subsequently, the yield of chickpea varieties increased by 40–65% (Khan and Rehman 1997). Soil application of a *T. virens* pellet formulation controlled damping-off caused by *R. solani* (Papavizas and Lewis 1989). Coating seeds with *T. harzianum*, *T. viride*, and *T. virens* significantly controlled *F. oxysporum* f. sp. *ciceri* wilt by 30–46%, and integration of biocontrol agent and carboxin increased seed yield by 25–43% (Dhedhi et al. 1990).

Helminthosporium (Bipolaris) causes leaf spot disease in cane palm, *Chrysalidocarpus lutescens,* and losses could reach 90% during rainy weather conditions. Field experiments were carried out to test the efficacy of seed treatment of cane palm against *Helminthosporium* infection. Isolates of *T. harzianum* and *T. viride* obtained from soil and having antagonistic activity against *Helminthosporium* were used in field trials. Seed treatment with spore suspension completely eliminated the disease and also significantly increased seed germination, seedling growth, and seedling vigor (Jegathambigai et al. 2009). Commercial formulations of *T. harzianum* (Plant Guard and Biocide) successfully controlled *F. solani*, *F. oxysporum*, and *Macrophomina phaseolina*, the main pathogens of root rot disease in grapevines. A complete elimination of these pathogens was recorded with Plant Guard, and a 51 and 48% increase in yield/vine was recorded with Plant Guard and Biocide, respectively (Riad et al. 2010). In another study, black rot caused by *Thielaviopsis paradoxa* in pineapple was controlled by *T. harzianum* (Wijesinghe et al. 2009).

A good deal of work has been conducted in field trials of *Aspergillus niger* against soil-borne fungal pathogens. In a field where muskmelon and watermelon crops were suffering from *Fusarium* wilt (sometimes *R. solani* and *Pythium* spp. were associated with the disease), treatment of seeds with *A. niger* (Kalisena SD) at 8 g/kg and soil with *A. niger* (Kalisena SL) at 30 g/pit resulted in 81% control of the disease. The vines were more vigorous, and even with 15% incidence of disease, yield was approximately 5% greater as compared to that in disease-free areas (Chattopadhyay and Sen 1996). Seed treatment with Kalisena SD also provided 30% less sheath blight disease over control plants (Kumar and Sen 1998). Problems of pre-and postemergence damping-off incited by *P. aphanidermatum* and *R. solani* in fruit and vegetable farms were successfully overcome by a combined treatment of seed and soil application of Kalisena SD and Kalisena SL (Majumdar and Sen 1998). Similarly, 93% control of charcoal rot of potato in a *Macrophomina phaseolina*-infested field was obtained with *A. niger* (Kalisena SD and Kalisena SL) (Mondal 1998). Winter sorghum can be strongly damaged by *Macrophomina* infection; however, *A. niger* (Kalisena SD) seed treatment brought down incidence of the disease from 30 to 7% (Das 1998).

Many filamentous fungi and yeasts have been shown to be effective antagonists of fungi infecting the aerial parts of plants (Blakeman and Fokema 1982; Blakeman 1985). Hyseik et al. (2002) reported that a *T. harzianum*-based commercial product (Supresivit) applied at 0.5 g/kg of mineral fertilizers could suppress foliage diseases in wheat, barley, maize, oil rape, and potato, and therefore increase yields. Several foliar diseases have also been reduced significantly (by more than 50%) when leaves were sprayed with spores of common phylloplane fungi, e.g., *Alternaria, Cochliobolus, Septoria, Colletotrichum*, and *Phoma* or with spores of hyperparasites (Omar and Heather 1979). Examples include the cucumber powdery mildew fungus *Sphaerotheca fuliginea* treated with spores of *Ampelomyces quisqualis* or *Tilletiopsis* (Hijwegen 1986), the wheat leaf rust fungus *Puccinia triticina* with spores of *Darluca filum* (Devay 1956), and the carnation rust fungus with *Verticillum lecanii* (Fleming 1980). Similarly, spraying a spore suspension of common bark saprophytes such as *Cladosporium* sp. and *Epicoccum* sp. (Fokkema 1971), and *Trichoderma* spp. on pruning cuts of fruit trees has prevented infection by canker-causing pathogens such as *Nectria galligena* and *Leucostoma* (*Cytospora* sp.). A spray with *Trichoderma* in the field reduced *Botrytis* rot of strawberries and grapes at harvest and in storage (Dubos and Bulit 1981) and dry eye rot of apple fruits (Tronsmo 1986). Andrews et al. (1983) showed that *Chaetomium globosum* was able to control scab (*Venturia inaequalis*) development when applied to apple leaves under experimental conditions.

Postharvest rot of several fruits could be reduced considerably by spraying the fruit with spores of antagonistic fungi and saprophytic yeasts at different stages of fruit development, or by dipping the harvested fruits in a spore suspension. Control of postharvest diseases caused by *B. cinerea* and *A. alternata* of apple and tomato has been successful by using culture filtrates of *T. harzianum* T22 (Ambrosino et al. 2005). Yeast such as *Metschnikowia pulcherrima* (Irina et al. 2006) reduced postharvest rotting of peach and apricot. Also, significant reduction of citrus green

mold (*Penicillium digitatum*) was obtained by treating fruits with antagonistic yeasts or the fungal antagonist *T. virens* (Zamani et al. 2006), whereas post-harvest *Botrytis* rot of strawberries or grapes was reduced by several sprays of *Trichoderma* spores on blossoms and young fruits (Sesan et al. 1999). Postharvest black rot caused by *Thielaviopsis paradoxa* of pineapple fruit has been controlled by *T. harzianum* (Reyes et al. 2004; Wijesinghe et al. 2009). *Penicillium* rot of pineapple was reduced considerably by spraying fruits with nonpathogenic strains of the pathogen (Singh et al. 2009). Similarly, several antagonistic yeasts protected grapes and tomatoes from *Botrytis*, *Penicillium*, and *Rhizoctonia* rots (Janisiewicz and Jeffers 1997). The film-forming *Saccharomyces cerevisiae* strain M25 showed a significant ability to reduce postharvest decay in apples caused by the phytopathogenic fungus and patulin-producer *Penicillium expansum* (Ortu et al. 2005). One such yeast, *Candida saitoana*, controlled postharvest decay of apples by inducing systemic resistance while at the same time increasing chitinase and β-1,3-glucanase activities in the fruit (El Ghaouth et al. 2003).

Bioinoculants in IPM

Some bioincoulants, especially *Trichoderma* spp., have been found to be quite compatible with common fungicides and nematicides such as Thiram, Vitavax, Carbendazim, Nemacur, and Furadon; hence, they can be used in integrated disease management programs. Chickpea and lentil seeds treated with *T. virens* (10^7 conidia/ml) and subsequently with 0.1% carboxin effectively reduced soil-borne populations of *F. oxysporum*, *R. solani*, and *Sclerotium rolfsii* (Mukhopadhyay et al. 1992). In the field, integrated use of *T. harzianum* with fungicidal seed treatments significantly reduced incidence of chickpea wilt complex and increased crop yields. Bean seeds sown in soil heavily infested with *B. cinerea*, *R. solani*, and *P. ultimum* and treated with conidia of the transgenic *Trichoderma* strain germinated, but the seeds treated with wild-type spores did not germinate (Brunner et al. 2005). Transgenic strain SJ3-4 of *T. atroviride* not only exhibited threefold greater inhibition of spore germination of *Botrytis cinerea* but also overgrew and caused lysis of *R. solani* and *P. ultimum* (Brunner et al. 2005). Seed treatment with Vitavax and Ziram resulted in 30% disease control. Disease control increased to 63% when *T. harzianum* was applied with the fungicides (Kaur and Mukhopadhyay 1992).

Bacterial Diseases and Their Management

The first evidence of bacteria being responsible for plant diseases was reported in 1982 when the association of a bacterium (now known as *Erwinia amylovora*) was established with fire blight disease of pear. Since then, numerous plant pathogenic bacteria have been identified. The bacteria that cause diseases in plants are facultative saprophytes and can be grown artificially on nutrient media; however, fastidious vascular bacteria are difficult to grow in artificial media and some do not grow in culture (Agrios 2005). Plant pathogenic bacteria are rod-shaped, the only exception

being *Streptomyces*, which is filamentous. *Streptomyces* produce spores, called conidia, at the end of the filament. Other bacteria, however, do not produce spores. Bacterial pathogenicity depends primarily on spore/conidia production in the shortest possible time. Bacterial diseases of plants occur at any location that is reasonably moist and warm. Under favorable environmental conditions, they may be destructive in any geographical region.

Plant pathogenic bacteria induce different kinds of symptoms in plants depending on causal agent and host, such as leaf spots and blights; soft rots of fruits, roots, and storage organs; wilts; overgrowths; scabs; and cankers (Fig. 17.3), and cause severe yield losses. There are eight major bacterial genera that are plant pathogenic and cause significant economic losses to plants: *Pseudomonas*, *Xanthomonas*, *Erwinia*, *Agrobacterium*, *Clavibacter*, *Curtobacteium*, *Rhodococcus*, and *Streptomyces* (Singh 2008). Bacterial canker of tomato is distributed throughout the world and may cause up to 60% yield loss (Chang et al. 1992). Yield reduction due to other important bacterial diseases may reach 5–25% (bacterial blight of cotton, Verma 1995), 6–60% (bacterial leaf blight of rice, Srivastava and Rao 1966), 10–15% (bacterial blight of mango, Kishun 1987), 8–16% (bacterial spots of chilli and tomato, Singh 2008), 10–70% (bacterial brown rot and wilt of potato, Verma and Shekhawat 1991), and 11–91% (bacterial wilt of tomato and eggplant, Kishun 1987).

Data on control of plant pathogenic bacteria with the application of bioinoculants is limited; however, a few studies conducted thus far have shown that bacterial

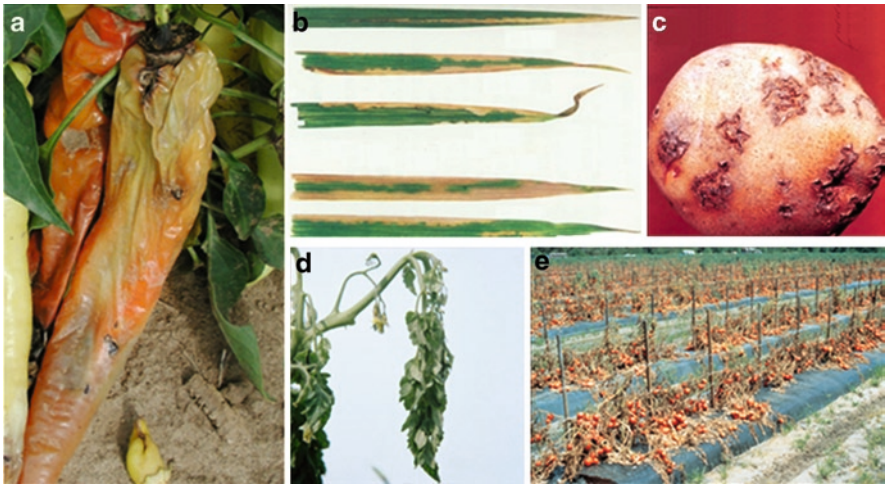

Fig. 17.3 Symptoms of some common plant diseases caused by bacteria. (**a**) Soft rot of chilli caused by *Erwinia carotovora* subsp. *Carotovora.* (**b**) Bacterial leaf blight of rice caused by *Xanthomonas oryzae* pv. *oryzae*, (**c**) Potato scab caused by *Streptomyces scabies*, (**d**) Bacterial wilt of tomato caused by *Ralstonia solanacearum* (symptoms on youngest leaves), (**e**) Bacterial wilt of tomato in the field, (Courtesy photo: (**a**) http://www.omafra.gov.on.ca/.../bacterial-soft-rot.html; (**b**) http://www.jxny.com/bctk/2009-4-15/sdbykb.htm; (**c**) http://www.hort.uconn.edu/ipm/veg/htms/scabpot.htm; (**d**) University, USDA Cooperative Extension Slide Series, Bugwood.org; (**e**) Courtesy J. P. Jones (http://www.apsnet.org/.../bacteria/text/fig02.htm))

diseases of plants can be successfully managed with bacterial antagonists. For example, bacterial crown gall has been controlled by treating seeds or nursery stock with bacteriocin-producing strain *Agrobacterium radiobacter* K-1026 (Jindal 1990). Some information is also available on management of bacterial plant pathogens with fungal bioinoculants. Treatment of tubers and seeds with fungal antagonists has proved effective against plant pathogenic bacteria, but not under field conditions (Agrios 2005). Kalita et al. (1996) reported 47.5% reduction in citrus canker incidence (*Xanthomonas campestris* pv. *citri*) after application of a strain of *Aspergillus terreus*. Bacterial wilt of tomato (*Ralstonia solanacearum*) and soil populations of the pathogen were reduced by soil application of *Glomus mosseae* together with *P. fluorescens* (Kumar and Sood 2002).

Nematode Diseases and Their Management

Parasitic nematodes are considered important pathogens of agricultural crops. Nematodes damage plants by injuring and feeding on root hairs, epidermal cells, cortical, and/or stelar cells (Khan 2008). A significant number of nematodes like *Rotylenchus*, *Hoplolaimus, Helicotylenchus*, *Tylenchorhynchus*, *Belonolaimus*, *Trichodorus*, and *Longidorus* are ectoparasites, which feed on the root surface. However, a considerable number of nematodes fully enter the host root and are termed endoparasites. Examples include root-knot nematodes (*Meloidogyne* spp.), cyst-forming nematodes (*Heterodera* spp.), and root-lesion nematode (*Pratylenchus* spp.). Some nematodes such as citrus nematode (*Tylenchulus semipenetrans*) and reniform nematode (*Rotylenchulus reniformis*) are considered semi-endoparasites as they only partly enter the host tissue.

Nematodes are documented to cause up to 7–12% yield loss to various crops. Yield losses vary greatly, depending on inoculum level and host species. Severe infection may result in as much as 80–90% yield decline in an individual field, and sometimes, plants fail to produce any yields of economic value. Nematode damage usually remains hidden and is not recognized by growers or scientists. This is not always the case, however. When fields are heavily infested, characteristic symptoms appear on roots or shoots. Specific symptoms include root lesions, root rot, root pruning, root galls, and cessation of root growth (Fig. 17.4).

Some nematodes also cause characteristic symptoms on aboveground parts. *Aphelenchoides* spp. cause necrosis and whitening of leaves of chrysanthemum, strawberry, and rice. *Ditylenchus dipsaci* attacks bulbs as well as buds of tulip and lily (Fig. 17.5). In addition to direct damage, nematodes often aid or aggravate diseases caused by fungi, bacteria and viruses or may break the resistance of cultivars to pathogens. Hairy root of rose, caused by *Agrobacterium rhizogenes*, is of minor importance, but in the presence of *Pratylenchus vulnus*, the disease becomes severe (Sitaramaiah and Pathak 1993). Fusarium wilt-resistant cultivars of cotton become susceptible in the presence of root-knot nematodes (Atkinson 1892). The degree of crop damage, however, depends largely on plant species or cultivar, nematode species, level of soil infestation, and prevailing environmental conditions.

Fig. 17.4 Symptoms of some common nematode diseases on roots. (**a**, **b**) Root lesion of tobacco caused by *Pratylenchus penetrans*, (**c**) Root-knot disease of tomato caused by *Meloidogyne incognita*, (**d**) Cysts of *Globodera rostochiensis*, the golden nematode on potato roots, (**e**) Blistered and cracked onion bulbs caused by *Ditylenchus dipsaci.* [Courtesy photo: (**a**) R. J. Reynolds Tobacco Company Slide Set, R. J. Reynolds Tobacco Company, Bugwood.org (http://www.forestryimages.org/.../3072x2048/1402035.jpg); (**b**) *C. C. Russell* (http://www.nematode.unl.edu/extpubs/kanfig3e.jpg); (**c**) *R. S. Hussey,* (http://www.apsnet.org/.../images/fig08.jpg); (**d**) courses.cit.cornell.edu/.../Golden_nematode.html; (**e**) http://www.inra.fr/.../HYPPZ/RAVAGEUR/6ditdip.htm]

Plant nematodes may also act as vectors for bacteria, fungi, and viruses. For instance, *Anguina tritici* carries *Clavibacter tritici* and *Dilophospora alopecuri* to the shoot meristem of wheat (Khan and Dasgupta 1993).

Biological control of nematodes may be achieved with two kinds of microorganisms, i.e., classical parasites or predators, and plant growth-promoting (PGPR) microorganisms. Classical parasites or predators such as *Paecilimyces lilacinus*, *Dactylaria candida*, and *Pasteuria penetrans* have been used in nematode control during the last few decades and reduce nematode population by direct action (De Bach 1964). PGPR may suppress rhizoshpheric nematode populations by promoting host growth, inducing systemic resistance, and/or producing nematoxic metabolites such as bulbiformin (Brannen 1995), phenazin (Toohey et al. 1965), and pyoleutorin (Howell and Stipanovic 1980).

In recent years, considerable research has been carried out on the use of bioinoculants to control nematode populations in soil. Effects of microorganisms have

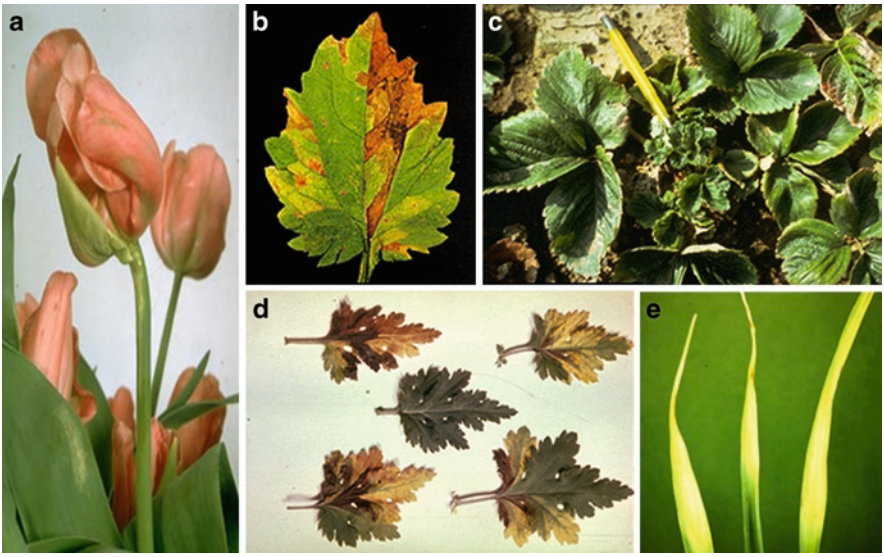

Fig. 17.5 Some common foliar symptoms of nematode diseases. (**a**) Damage to tulip flower due to *Ditylenchus dipsaci*, (**b**) *Aphelenchoides ritzemabosi* on chrysanthemums, (**c**) Cauliflower disease of strawberry caused by *Aphelenchoides fragariae*, (**d**) *Aphelenchoides fragariae* on carnation leaf, (**e**) White tip of rice caused by *Aphelenchoides besseyi*. [Courtesy photo: (**a**) Central Science Laboratory, Harpenden Archive, British Crown, Bugwood.org (http://www.forestryimages.org/browse/detail.cfm?imgn); (**b**) http://www.floranazahrade.cz/poradna/poradna2003_12.htm; (**c**)ucdnema.ucdavis.edu/.../204NEM/2FOLIAR.htm;(**d**)ucdnema.ucdavis.edu/.../204NEM/2FOLIAR.htm; (**e**) Donald Groth, Louisiana State University AgCenter, Bugwood.org (http//www.forestryimages.org/browse/detail.cfm?imgn)]

been evaluated against different nematodes under in vitro, pot, and field conditions. Some important fungal bioinoculants are listed in Table 17.2.

In Vitro Studies

Culture filtrate of *Pochonia chlamydosporia* in yeast extract medium has demonstrated pronounced nematicidal and nematostatic effects. A dilution of 1:1 culture filtrate resulted in 100% mortality of *Globodera rostochiensis*, *G. pallida*, and *Panagrellus redivivus* (Saifullah 1996c). Strains of *T. virens* and *Burkholderia cepacia* (bacteria) were found to produce extracellular factors that decreased *M. incognita* egg hatch and juvenile mobility (Siddiqui and Shaukat 2004). Eapen and Venugopal (1995) have shown that isolates of *Trichoderma* spp. have broad-spectrum biocontrol activity against a number of pathogenic fungi and nematodes. A serine protease of 28 kDa with trypsin activity was isolated from *Trichoderma* strain 2413. The enzyme reduced the number of hatched eggs of root knot nematodes and showed synergistic effects with other proteins produced during antagonistic activity of the strain (Benitez et al. 2004). The number of hatched eggs of the root-knot nematode, *M. incognita,* was significantly reduced after incubation with

Table 17.2 Effect of different bioinoculant fungi on plant nematodes infesting agricultural crops

Antagonistic fungi	Nematode managed	Host plant	References
Aspergillus niger	*Meloidogyne* spp.	Tomato	Singh et al. (1991)
A. niger	*M. incognita*	Okra	Sharma et al. (2005)
A. niger	*M. incognita*	Eggplant	Khan and Anwer (2008)
A. niger	*M. incognita*	Tomato	Khan et al. (2007)
Paecilomyces lilacinus	*Meloidogyne* spp.	Tomato	Khan and Tarannum (1999); Pal and Gardener (2006); Schenek (2004)
P. lilacinu and *T. virens*	*M. incognita*	Tomato	Khan and Akram (2000)
P. lilacinus and *P. chlamydosporia*	*M. incognita*	Mung bean	Khan and Kounsar (2000)
P. lilacinus	*M. javanica*	Tobacco	Hewlett et al. (1988)
P. lilacinus	*R. reniformis*	Tomato	Lysek (1966)
P. lilacinus	*Meloidogyne* spp.	Various	Jatala (1986)
P. lilacinus	*Meloidogyne* spp.	Okra	Khan and Ejaz (1997)
Penicillium anatoticum	*Globodera* sp.	Potato	Jatala (1986)
T. harzianum, P. lilacinus	*M. incognita*	Chickpea	Pant and Pandey (2002)
T. harzianum	*M. javanica*	Tomato	Siddiqui and Shaukat (2004)
T. harzianum	*M. arenaria,*	Corn	Windham et al. (1989)
T. harzianum	*Meloidogyne* spp.	Cardamom	IISR 1995
Trichoderma asperellum -203 and *Trichoderma atroviride*	*M. javanica*	In vitro	Sharon et al. (2009)
T. pseudokoningii, T. viride, P. lilacinus, A.niger, G. mosseae	*M. incognita*	Soybean	Oyekanmi et al. (2008)
T. harzianum, P. lilacinus	*Meloidogyne javanica*	Okra	Zareen et al. (2001)
T. atroviride	*R. similis*	Banana	Zum Felde et al. (2006); Pocasangre Enamorado et al. (2007)
T. harzianum (T014)	*M. incognita*	Gladiolus	Khan and Mustafa (2005)
T .harzianum and *P. chlamydosporia*	*Globodera rostochiensis* and *G. pallid*	Potato	Saifullah (1996a, b)
T. harzianum and *P. chlamydosporia*	*M. incognita*	Chickpea	Khan et al. (2005a)
T. harzianum and *P. chlamydosporia*	*H. cajani*	Pigeonpea	Siddiqui and Mahmood (1996)

pure PRA1 (trypsin-like protease) preparations of *T. harzianum* CECT 2413 (Suarez et al. 2004). In another study, *T. asperellum*-203 *and T. atroviride* suppressed *M. javanica* populations by direct effect on various developmental stages of nematodes, viz., eggs, larvae, and adults (Sharon et al. 2009). Culture filtrates of *Aspergillus niger* soil isolates AnC2 and AnR3 efficiently suppressed hatching of eggs and mortality of juveniles of *M. incognita* (Khan and Anwer 2008).

Pot Conditions

The majority of studies exploring the potential of bioinoculants against plant nematodes have been carried out under pot conditions (Khan 2007). In a pot experiment, chilli (*Capsicum annum*) seedlings were inoculated with *Meloidogyne javanica*, *Aspergillus niger*, and *Rhizoctonia solani* alone or in various combinations. All growth parameters were significantly greater with *A. niger* and lower with *M. javanica* or *R. solani* (Shah et al. 1994). Singh et al. (1991) showed that application of *A. niger* decreased the damage caused by *M. incognita* and *R. solani* singly or together on the tomato cultivar, Perfection. Similarly, inoculation with *A. niger*, *Epicoccum purphurascum*, *Penicillium vermiculatum*, and *Rhizopus utgricans* effectively diluted the adverse effect of *R. solani* and *M. incognita* resulting in an increase in germination of the tomato cultivar, Pusa Ruby (Rekha and Saxena 1999). In a pot experiment, application of *A. niger* isolates (AnC_2 and AnR_3) significantly suppressed galling, egg mass production, and soil populations of *M. incognita*. The isolates AnC_2 and AnR_3 produced the greatest quantities of siderophores, HCN and NH_3, and solubilized the greatest quantity of soil phosphorus (Khan and Anwer 2008). Windham et al. (1989) reported a suppressive effect of *T. harzianum* on *M. arenaria* resulting in an increase in root fresh weight and decrease in number of eggs per gram of root. Significant reduction in *H. avenae* populations and increase in wheat growth were recorded with *P. chlamydosporia* (Bhardwaj and Trivedi 1996). In another study, application of the same fungus decreased the number of eggs, juveniles, and galls of *M. hapla* in tomato plants (De leij et al. 1993).

Application of *T. virens* and *Burkholderia cepacia* (bacteria) as a seed coat followed by root drenches suppressed root-knot nematode infestation in bell pepper compared with untreated plants (Meyer et al. 2000). Pant and Pandey (2001) reported maximum reduction in populations of *M. incognita* with *T. harzianum*, *P. lilacinum*, and *A. niger* applied in sterilized soil in pots at 5,000 spores/pot. In a greenhouse test, *P. chlamysosporia* provided 75% control of the first cropping of *Heterodera schachtii.* Ashraf and Khan (2008) evaluated the efficacy of wastes of apple (*Malus pumila*), banana (*Musa paradisiaca*), papaya (*Carica papaya)*, pomegranate (*Punica granatum*), and sweet orange (*Citrus sinesis*) at 20 g/plant and *Paecilomyces lilacinus* at 2 g (mycelium + spores)/plant against the reniform nematode, *Rotylenchulus reniformis,* on chickpea. The best protection of chickpea against *R. reniformis* was recorded using integration of *P. lilacinus* with papaya wastes, followed by apple and pomegranate wastes. Control of *M. javanica* was accomplished by inoculating soil with *P. chlamydosporia*-colonized rice medium at a rate of 30 g/kg soil (De leij et al. 1993). Introduction of the fungus 2 weeks before nematode inoculation provided significantly greater control of *M. javanica* (De leij et al. 1993). Application of culture filtrate of *T. harazianum, T. viride, T. koningii, T. reesei* and *T. hamatum* resulted in effective control of the reniform nematode (*Rotylenchulus reniformis*) and root-knot nematode (*M. javanica*) on the eggplant cultivar, Black Beauty (Bokhari 2009).

Field Conditions

Relatively few field trials have been conducted to evaluate the effectiveness of bioinoculants against nematode infestations. These studies, however, have demonstrated that nematode control to a level can be exploited commercially (Khan 2005). Soil treatment by *A. niger* in castor beans abated the population of *Rotylenchulus reniformis* up to 71% (Das 1998). Suppression of root-knot nematodes resulting in improved growth of cardamom seedlings in nurseries due to application of *T. harzianum* has been reported (IISR 1995). *Pochonia chlamydosporia* var. *catenulate* integrated with other strategies reduced soil populations of plant parasitic nematodes (51–78%) in vegetable crops (Garcia et al. 2004). Under natural soil conditions, nematode eggs appear to be an important source of nutrients for *P. chlamydosporia.* The fungus parasitized large numbers of *H. avenae* eggs in English cereal fields and played a major role in limiting multiplication of the nematode (Kerry et al. 1982a, b). In a field experiment, effects of root-dip treatment of ornamental plants hollyhock (*Althea rosea*), petunia (*Petunia hybrida*), and poppy (*Papaver rhoeas*) with *P. chlamydosporia*, *P. fluorescens*, and *B. subtilis* were evaluated. The three bioinoculants suppressed galling of *M. incognita* by 37%, 27%, and 24%, respectively (Khan et al. 2005b). Chlamydospores of some biotypes of *P. chlamydosporia* applied to soil significantly reduced (>50%) population densities of *M. hapla* on tomato and of *G. pallida* on potato plants (Siddiqui et al. 2009). In another study, Kumar (2009) reported satisfactory control of root knot of papaya with *P. chlamydosporia.*

Soil application of *Paecilomyces lilacinus* with or without neem leaf powder reduced galling and egg mass production by 24–46% and enhanced yield of okra by 15% (Khan and Ejaz 1997). In another study, soil application or root dip treatment of tomato seedlings with *Bacillus subtilis* or *Pseudomonas stutzeri* controlled root knot of tomato (Khan and Tarannum 1999). Application of *P. fluorescens, T. virens*, or *P. lilacinus* controlled the root knot caused by of *M. incognita* in the presence or absence of wilt fungus, *Fusarium oxysporum* f. sp. *lycopersici* (Khan and Akram 2000; Akram and Khan 2006). A field study conducted to evaluate relative effectiveness of seed treatment with different rhizobacteria (*Azotobacter coccum, Azospirillum lycopirum*, *B. subtilis*, and *Bijrica indica*) and antagonistic fungi (*Arthrobotrys oligospora*, *Cylindrocarpon destructans*, *Pochonia chlamydosporia*, and *P. lilacinus*) on root nodulation and plant growth of green gram revealed that treatment with *B. subtilis* or *B. indica* reduced galling by 33–34% and increased dry weight of shoots by 22–24% (Khan and Kounsar 2000; Khan et al. 2002). Other bioinoculants were also found to be effective. Seed treatment with *P. fluorescens* or *B. subtilis* was effective against root knot of green gram (Khan et al. 2007).

Siddiqui and Shaukat (2004) reported that combined application of *T. harzianum* with *P. fluorescens* in unsterilized sandy loam soil caused significant reduction in *M. javanica* population densities in tomato roots. Application of *P. chlamydosporia* at 20 g/plot (6×10^7 cfu/g substrates) along with *P. lilacinus* and neem cake effectively controlled *M. incognita* and increased yield (58%) of inoculated brinjal plants (Cannayane and Rajendran 2001). Dhawan et al. (2008)

reported that combined effects of *P. chlamydosporia* and *P. fluorescens* significantly managed the root-knot nematode *M. incognita* and increased yield of brinjal in farmer's fields. Bioefficacy and compatibility of formulations of *P. chlamydosporia* (2×10^6 cfu/g) and *P. lilacinus* (2×10^6 cfu/g) were evaluated against the root-knot nematode *M. javanica* infecting nursery of acid lime. Application of 5 or 10 g of each bioinoculant formulation and combined use of *P. lilacinus* and *P. chlamydosporia,* each at 10 g/kg soil, significantly reduced root-galling index and number of nematodes in roots (Rao 2005).

17.1.2 Production Technology of Bioinoculants

For field application of a bioinoculant, an efficient substrate for mass production and an inert immobilizing material are required, which could carry the maximum number of propagules of the organism with minimum volume and necessarily maintain its survival and integrity. An excellent bioinoculant is one that is introduced into an ecosystem, and subsequently survives, proliferates, becomes active, and establishes itself in a new environment (Khan 2005). For preparing a commercial formulation, these attributes must be considered. In addition, the bioinoculant should be mass cultured on an inexpensive substrate in a short period of time. Easy application, effectiveness, and consistent results under a variety of environmental conditions are other desirable features required for production of bioinoculant formulations.

Different techniques of cell immobilization have been developed to devise efficient carrier systems to produce commercial formulations of bioinoculants. A number of carriers for immobilization of microorganisms have been used to develop commercial formulations of biocontrol agents, viz., peat, seeds, meals, kernals, husks, bran, bagasse, farmyard manure, cow dung cake, compost, oil cakes, wood bark, vermiculite, sand, clay, and liquid carriers. Three types of formulations, viz., pellet, granular, and liquid, are widely produced.

17.1.2.1 Pellet Formulations

A small amount of liquid bioinoculant culture encapsulated by some appropriate inert material to hold the suspension and organism intact is termed a pellet. Different materials such as natural polymers (alginate, carrageenan, cellulose, agar, agarose, hen-egg white, gelatin) as well as synthetic polymers (polyacrylamine, photo cross-linkable resins, etc.) can be used to encapsulate liquid suspension of bioinoculants to formulate efficient delivery systems for field application of microorganisms (D'Souza and Melo 1991). The gelant sodium alginate is considered a useful material for encapsulation of liquid preparations of microorganisms. The microbes remained viable for many weeks in alginate pellets. Fravel et al. (1985)

prepared pellet formulation on a comminuted (blended) mixture of sodium alginate and pyrax (pyrophyllite, hydrous aluminum silicate) in a 1:10 ratio. The mixture was amended with bioinoculant liquid suspension in the ratio 9:1. The alginate–pyrax–microorganism mixture was stirred continuously while dripping through a pipette into a solution of 0.25 M $CaCl_2$ or 0.1 M $C_{12}H_{22}CaO_{14}$ (calcium gluconate). The pellets, after drying under sterile air (laminar flow hood), were stored at different temperatures for various durations in a deep freezer to determine viability of the spores. Populations of the microorganism pellets were determined by the dilution plate method. Some pellet formulations are listed in Table 17.3.

Successful encapsulation of liquid suspension of spores and hyphae of *P. chlamydosporia* was conducted with sodium alginate containing 10% (w/v) kaolin or wheat bran (De Leij and Kerry 1991). On soil application, the fungus proliferates in soil from those granules which contained wheat bran as the energy source. In another study, Kerry (1988) estimated approximately 9×10^4 and 4×10^4 cfu of *P. chlamydosporia*/g soil after 1 and 12 weeks of application of granules, respectively.

17.1.2.2 Powder Formulations

Granular or powder carrier systems for microorganisms are more useful than pellets and are compatible with existing farm machinery. Formulations of fungal bioinoculants can be successfully prepared on fermenter biomass in the form of powder with diluents such as cake (semisolid), pyrax, or alginate pellets containing a food base such as bran (Papavizas et al. 1984; Beagle-Ristaino and Papavizas 1985). Papavizas and Lewis (1989) prepared two formulations of *T. virens*, alginate–bran–fermenter biomass pellets and pyrax–fermenter biomass mixture. The formulations were available at low cost as they were developed from inexpensive agriculture/industrial wastes or by-products. A good immobilizing material is one that provides an energy base for the sustenance and multiplication of the bioinoculants. Numerous powder/granular formulations have been prepared and marketed (Table 17.4).

Liquid stillage, a by-product of sorghum fermentation, can be added to granular lignite in a 1:2 ratio and stirred (Jones et al. 1984). The amended granules are dried overnight at 30°C, treated again with the stillage (50% v/v), and autoclaved in conical flasks or polyethylene bags. The sterilized mixture is inoculated with a liquid suspension of fungal bioinoculants such as *T. harzianum* and *T. virens*. Four days after inoculation at 25°C, the flasks/bags are shaken to distribute evenly the sporulating fungus. Populations of the microorganism pergram of granules and their viability with regard to storage temperature and duration are determined by the dilution plate method. The air-dried granules can be prepared and stored at 20°C for up to 4 months with 90% viability of spores.

Various agricultural materials, industrial wastes, and by-products, viz., wheat bran–sand mixture, sawdust–sand–molasses mixture, corn cob–sand–molasses mixture, bagasse–sand–molasses mixture, organic cakes, cow dung–sand mixture, compost/farm manure, inert charcoal, diatomaceous earth, and fly ash can also be used to prepare powder formulations of bioinoculants (Khan 2005).

Table 17.3 Some pellet formulations of fungal bioinoculants

Product name	Bioinoculant	Target pathogen(s)/disease(s)	Crop(s)	Company
AQ10 Biofungicide	*Ampelomyces uisqualis* isolate M-10	Powdery mildew	Various	Ecogen, Inc, USA
Trichopel	*T. harzianum, T. viride*	*Nectia, Phytophthora, Pythium*	Various	Agrimm Technology, New Zealand
SoilGard (GlioGard)	*Gliocladium* (*Trichoderma*) *virens GL-21*	Damping-off, *Pythium*, *Rhizoctonia solani*	Various	Certis, USA
F-Strop	*T. harzianum*	*Pythium, Rhizoctonia*	Various	USA.Reg. No. 68539-3
PlantShield, Planter box	*T. harzianum* T-22 *T. harzianum* KRL_AG2(T-22)	*Pythium, Rhizoctonia, Fusarium*	Various	Bioworks inc, USA
DiTera WDG	*Myrothecium verrucaria*	Parasitic nematodes	Various	Abbott laboratories, USA
Contans WG, Intercept WG	*Coniothyrium verrucaria*	*Slerotinia slerotiorum*, *S. minor*	Many crops	Abbott laboratories, USA
Aspire	*Candida oleophila* I-182	*Botrytis, Penicillium*	Citrus, pome fruit	Ecogen Inc, USA
Rootshield	*Trichoderma* spp.	*Pythium, Rhizoctonia, Phytophthora*	Various	BioWorks, India
T22-Planter Box	*T. harzianum*	*Pythium, Rhizoctonia, Fusarium*	Various	BioWorks, India
Kalasipahi	*Aspergillus niger* AN27	Soil-borne fungi	Various	Cadila Pharmac. Ltd., India

Table 17.4 Some powder formulations of fungal bioinoculants

Product name	Bioinoculant	Target pathogen(s)/disease(s)	Crop(s)	Company
Bio-Fungus	*Trichoderma* spp.	Soil-borne fungi	Various	Grondortsmettingen DeCuester n.v., Belgium
Biowilt-X	*T. harzianum*	*Fusarium* spp.	Pulses	Department of Plant Protection, Aligarh Muslim University, India
Bionem-X	*Pochonia chlamydosporia*	*Meloidogyne* spp.	Pulses, vegetables	Department of Plant Protection, Aligarh Muslim University, India
Trichodowels	*T. harzianum, T. viride*	*Pythium, Rhizoctonia, Fusarium*	Trees, ornamental.	Agrimm Technology, New Zealand
VinevaxTM (formerly Trichoseal)	*T. harzianum*	Various	Vines and trees	Agrimm Technology, New Zealand
Trichodex	*T. harzianum*	*Colletotrichum, Monilin., Plasmopara*	Various	Makhteshim chemical works ltd, USA
Rotstop	*Phlebia gigantean*	*Heterobasidion annosum,*	Trees	Verdera, USA
Shemer	*Metschnikowia fructicola*	*Botrytis, Rhizopus, Aspergillus, Penicillium*	Strawberry, grape, sweet potato, citrus	Minrav, Israel
BINAB T	*T. harzianum/T. polysporum*	Wood decay fungi	Trees	Binab bio-innovation, USA
RootShield	*T. harzianum* T-22	*Pythium, Rhizoctonia, Fusarium*	Various	Bioworks inc, USA
Primastop	*Gliocladium catenulatum*	Soil-borne pathogens	Various	Verdera oy, USA
Polygandron	*Pythium oligsndrum*	*Pythium ultimum*	Sugar beet	
DiTera wp	*Myrothecium verrucaria*	Parasitic nematodes	Various	Abbott laboratories, USA
Plant-Shield	*Trichoderma* spp.	Root diseases	Various	BioWorks, India.
Ecofit	*T. viride*	Root diseases	Various	Hoech. Scher. Agr. Evo. Ltd., India
Sanjeevni	*T. viride*	Seed and soil born diseases	Various	International Panaecea Ltd., India
Bioderma	*T. viride* + *T. harzianum*	*Phytophthora, Pythium, Rhizoctonia*	Various	Biotech International Ltd., India

Bas-derma	*T. viride*	Seed- and soil-borne diseases	Various	Banaras Biocontrol Res. Lab., India
Ecoderma	*T. viride*+*T. harzianum*	*Pythium, Rhizoctonia, Fusarium spp.,* parasitic nematodes	Various	Margo Biocontrol Pvt. Ltd., India
Defense-SF	*T. viride*	Various seed and soil born diseases	Various	Workhard Life Science Ltd., India
Kalisena SL Kalisena SD Pusa Mrida Beej Bandhu	*Aspergillus niger* AN27	*Fusarium oxysporum, F. solani, Macrophomina phaseolina, Pythium aphanidermatum, R. solani, Sclerotinia sclerotiorum* etc.	Various	Cadila Pharmac. Ltd., India
Funginil	*T. viride*	Seed and soil born diseases	Various	Crop Health Biop. Res. Cen. India
Manidharma's	*Paecilomyces lilacinus*	Parasitic nematodes	Various	Mani Dharma Biotech, India
Manidharma's Trichoderma sp	*Trichoderma harzianum, T. viride*	Root rots, Wilts, brown rot, damping-off, charcoal rot, soil born diseases	Various	Mani Dharma Biotech, India
Tricho-X	*T. viride*	Various soil born diseases	Various	Excel Industries Ltd., India
Trieco	*T. viride*	Seed- and soil-borne fungi, nematodes	Various	Ecosense Labs Pvt. Ltd., India
Tri-control	*Trichoderma* spp.	Various seed, soil and foliar diseases	Various	Jepee Biotech. India.
Sun Agro Derma	*Trichoderma viride*	Seed-, soil-borne and foliar diseases	Various	Bio Organic Industries, India
Sun Agro Derma–H	*Trichoderma harzianum*	Seed-, soil-borne and foliar diseases	Various	Bio Organic Industries, India
CHAETO	*Chaetomium globosum*	Spot blotch	Wheat, oats, barely	Bio Organic Industries, India
Sun nema	*Paecilomyces lilacinus*	Plant nematodes	Various	Bio Organic Industries, India
Trichoguard	*T. viride*	Various soil born diseases	Various	Anu Biotech International Ltd., India
Trichorich-WP	*Trichoderma viride*	Seed and soil born diseases	Various	Richgreen Agrochem, India
Vertirich-WP	*Verticillium lecanii*	Seed and soil born diseases	Various	Richgreen Agrochem, India
JaiVJai Bio-Tricure	*Trichoderma viride*	Various fungi	Various	Chaitra Fertilisers & Chemicals, India
Bioguard	*T. viride*	Seed and soil born diseases	Various	Krishi Rasayan Exp., Ltd., India

Backman and Rodriguez-Kabana (1975) prepared a commercial formulation of *T. harzianum* on sterilized granules of diatomaceous earth impregnated in 10% molasses for four days. The bioinoculant remained viable after air-drying for up to 1 month in cold storage. Kelley (1976) used clay granules with additional nutrients to produce *T. harzianum* formulations. Khan et al. (2001) used grains and meals of cereals, corn cob–sand–molasses, compost, leaf litter, bagasse–soil–molasses, and sawdust–sand–molasses to mass-culture *T. harzianum*, *T. virens*, and *P. chlamydosporia*. Highest cfu counts of *Trichoderma* spp. (10^{6-7} cfu/g material) and *P. chlamydosporia* (10^{5-6} cfu/g material) were recorded in bagasse–soil–molasses and leaf litter–sucrose, respectively. Cabanillas and Barkar (1989) tested wheat grains, alginate pellets, and diatomaceous earth granules to produce a commercial formulation of *Paecilomyces lilacinus* for soil application. The formulation contained active propagules of the antagonist in higher number, and the application effectively controlled root-knot disease in tomato and consequently increased yields.

Khan (2005) developed a novel process for production of commercial formulations by bioinoculants, viz., *T. harzianum*, *P. chlamydosporia*, and *P. fluorescens*. The process involved two steps: the first dealt with the preparation of mass culture or stock culture of the microorganisms on sawdust, soil, and 5% molasses mixture in the ratio of 15:5:1. The immobilization of the microorganisms took place on a fly ash-based carrier (fly ash, soil, and 5% molasses, 15:3:1). One part of the stock culture and 20 parts of the carrier were packed in a poly pack and incubated at 25°C for 1 week. Using the process, three commercial formulations of *T. harzianum* and *P. chlamydosporia* were prepared. The bioinoculants were found viable in the formulation up to 32 weeks at 25°C or at room temperature. Seed treatment or soil application of the formulations successfully carried the microorganisms to soil (field) and effectively controlled soil-borne fungi and nematodes on vegetables and pulse crops (Khan 2005).

17.1.2.3 Liquid Formulations

Single-stage liquid fermentation of fungal and bacterial bioinoculants is an attractive process from an industrial point of view, as sometimes it becomes difficult to improve production of conidia (spores) on solid materials (grains, powder, etc.). Several liquid media for fungal bioinoculants such as potato dextrose broth (PDB), Sabouraud dextrose broth with yeast extract (SDYB), Sabouraud maltose broth with yeast extract (SMYB), malt extract broth (MEB), corn meal broth (CMB), jaggery soya broth (JSB), yeast peptone dextrose broth (YPDB), yeast peptone soluble starch broth (YPSS), Czapek–Dox broth (CDB), and yeast peptone soybean oil broth (YPSB) in stationary and shaker culture have been evaluated for mass production of *Beauveria bassiana*, *Metarhizium anisopliae*, *T. harzianum,* and *T. viride*. Maximum biomass production of bioinoculants was observed with JSB in stationary (12.5–20/100 ml wet wt.) and shaker cultures (20–48.8 g/100 ml wet wt.), and highest cfu (5.1 and 9.8×10^8cfu/ml) in stationary and shaker culture were observed, respectively (Rao and Gopalakrishnan 2009). Some liquid formulations are listed in Table 17.5.

Table 17.5 Some liquid formulations of fungal

Product name	Bioinoculant	Target pathogen(s)/disease(s)	Crop(s)	Company
Trichojet	*T. harzianum, T. viride*	*Nectia, Phytophthora, Pythium, Rhizoctonia*	Various	Agrimm Technology, New Zealand
DiTera ES	*Myrothecium verrucaria*	Parasitic nematodes	Cole crops, grapes, ornamental, turf, trees	Valent biosciences corporation, USA
Fusaclean	Nonpathogenic *F. oxysporum*	*F. oxysporum*	Tomato, carnation, basil, cyclamen	Natural Plant Protection, Route d'Artix, France
T22-HC	*T. harzianum*	*Pythium, Rhizoctonia, Fusarium*	Various	BioWorks, India
Enpro-Derma	*Trichoderma viride*			
Filamen AQ	*Ampelomyces quisqualis*	Powdery mildew	Grape, Rose etc	Enpro Bio Sciences, India
Paecilon	*Paecilomyces lilacinus*	Nematode infestation in the soil	Pomegranate	Enpro Bio Sciences, India
Trichorich-L	*Trichoderma viride*	Seed and soil born diseases	Various	Richgreen Agrochem, India
Vertirich-L	*Verticillium lecanii*	Seed and soil born diseases	Various	Do
Bio Chemical Trichoderema	*Trichoderma viride*	Various fungi	Various	Ruchi Biochemicals, India

Peighami-Ashnaei et al. (2009) evaluated combinations of two carbon (sucrose and molasses) and two nitrogen (urea and yeast extract) sources for rapid growth and yield of *P. fluorescens* and *B. subtilis* and found that media containing molasses and yeast extract (MY) in a 1:1 w/w ratio supported rapid growth and high cell yields in both strains. Luna et al. (2002) and Peighami-Ashnaei et al. (2009) showed that maximum growth of the two bioinoculants was obtained when the C/N ratio was 1:1. Molasses is a high quality and inexpensive substrate and can be used for rearing bioinoculants through liquid fermentation. Substantial fungal biomass (spores + mycelia) was formed by incubating *Trichoderma* spp. on molasses in fermenter vessels for 15 days. The biomass was filtered, dried, milled, and mixed with anhydrous aluminum silicate as a diluent to increase volume for application (Papavizas et al. 1984). The filtered microbial biomass may also be formulated with selected liquids.

Bioinoculant formulations are often applied as drenches, spot treatments, or granules, but applying them as foliar sprays creates technical challenges. Use of oils may help to overcome this restriction of foliar application. The intermediate solution is to use more conventional formulations (e.g., wettable powder, WP) or technical materials (e.g., pure, dried fungal conidia) with emulsified oil adjuvants such as "Codacide" (Bateman and Alves 2000). However, as Wraight and Carruthers (1999) point out, oil formulations should be seen as a "silver bullet"; successful development will require a rigorous approach to selection of isolates, delivery system, and deployment in the marketplace.

17.2 Conclusion

Plant diseases are significant constraints on crop production worldwide, and their management is essential to increase food production. In view of the adverse effects of pesticides, fungal bioinoculants offer a potential substitute. Numerous potentially useful microorganisms are available, such as *Trichoderma* spp., *Aspergillus niger*, *Penicillium digitatum*, *P. anatolicum*, *Paecilomyces lilacinus*, *Pochonia chlamydosporia*, or nonpathogenic strains of certain pathogens. These organisms can be applied directly to soil, as a seed treatment or foliar spray to reduce the inoculum level of pathogen or disease severity. Commercial formulations of most bioinoculants are available and provide varied degrees of disease control. Overall performance of phosphate-solubilizing fungi such as *A. niger*, *Trichoderma* spp., *Penicillium* spp., against plant diseases and nematodes is at levels that ensure their commercial exploitation. This necessitates research efforts toward identification of more efficacious and environmentally adaptable strains, development of suitable mass production technologies, and development of efficient immobilization systems.

Bioinoculant formulations can be seen as a tool for developing a more rational pesticide use strategy. Understanding the implications of working with living organisms in agricultural systems is highly desirable. Perhaps more importantly,

biological control/IPM practitioners, organic growers, and other parties willing to promote bioinoculants must understand that they are most likely to succeed as commercial products, available as practicable, stable, efficacious formulations.

17.2.1 Future Recommendations

The use of bioinoculants is likely to become more widespread in the near future, as increasing pressure develops to limit environmental damage from the use of chemicals as well as development of pathogen resistance to pesticides. Environmentally sustainable systems for control of soil-borne pathogens are likely to be developed because the soil environment provides a more favorable habitat for the persistence of antagonists. In addition, the necessity for new systems will increase, requiring greater research efforts to develop technologies and methods for foliar application of bioinoculants. The technology available presently is able to produce liquid, powder, pellet, and granular formulations of bioinoculants, and limited formulations that are compatible for foliar application are available. Moreover, efficient methods are needed for improving multiplication rate of useful bioinoculants, which will enable bulk inoculum production with longer shelf life.

It is likely that genetically engineered microorganisms will be increasingly used in the future because it is often difficult to select, from natural microflora, an organism that both adapts to persist in the environment of roots or shoots of crop plants and possesses a high level of antagonistic activity against pathogens. Introducing a desired antagonistic ability, such as antibiotic or lytic enzyme production, into an organism that is both persistent and an effective colonist of roots or shoots may allow for such difficulties to be overcome. Such development must be combined with risk assessment studies to ensure the safety of the released bioinoculant, to provide adequate food to burgeoning populations, especially in Asia and Africa.

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Chapter 18
Mycorrhizal Inoculants: Progress in Inoculant Production Technology

Zaki A. Siddiqui and Ryota Kataoka

Abstract Of the seven types of mycorrhizae, the symbiotic association of plants with arbuscular mycorrhizae (AM) and ectomycorrhiza (ECM) is the most abundant and widespread. Mycorrhizal inoculant technology, especially of AM and ECM, appears to be a promising avenue for sustainable agriculture and forestry because of their extensive and productive association with plants. Production of mycorrhizal inocula is a complex procedure that requires commercial enterprises to develop the necessary biotechnological skill and ability to respond to legal, ethical, educational, and commercial requirements. At present, commercial mycorrhizal inocula are produced in pots, nursery plots, containers with different substrates and plants, and aeroponic systems, and by nutrient film technique, or in vitro. Different formulated products are now marketed, which creates the need for the establishment of standards for widely accepted quality control. Generally, preparation and formulation of mycorrhizal inocula are carried out by applying polymer materials with well-established characteristics and which are useful for agriculture and forestry. The most commonly used methods involve entrapment of fungal materials in natural polysaccharide gels, which includes immobilization of mycorrhizal root pieces, vesicles, and spores, in some cases coentrapped with other plant-beneficial microorganisms. Efforts should be devoted toward registration procedures of mycorrhizal inoculants to stimulate the development of mycorrhizal products industry. Biotechnology research and development in such activities must be encouraged, particularly with regard to interactions of mycorrhizal fungi with other rhizosphere microbes, and selection of new plant varieties with enhanced mycorrhizal traits to provide maximum benefits to agriculture and forestry.

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_18,

18.1 Introduction

Mycorrhizal fungi form symbiotic relationships with plant roots in a fashion similar to that of root-nodule bacteria of legumes. Of the seven types of mycorrhizae documented (arbuscular, ecto-, ectendo-, arbutoid, monotropoid, ericoid, and orchidaceous mycorrhizae), arbuscular mycorrhizae and ectomycorrhizae are the most abundant and widespread (Smith and Read 1997; Allen et al. 2003). Arbuscular mycorrhizal (AM) fungi comprise the most common mycorrhizal association and form mutualistic relationships with over 80% of all vascular plants (Brundrett 2002). Ectomycorrhizal (ECM) fungi are also widespread in their distribution but are associated with only 3% of vascular plant families (Smith and Read 1997). These two groups of mycorrhizal fungi play an important role in sustainable agriculture and forestry (Siddiqui and Mahmood 1995; Akhtar and Siddiqui 2008; Futai et al. 2008; Siddiqui and Pichtel 2008; Akhtar et al. 2011). The production of commercial inocula of these fungi has been increasing, particularly in the last few years, due to the following:

1. Their positive impact on plant health and development, land reclamation, phytoremediation, and disease management,
2. Increased awareness about biodiversity, concerns about soil microbial communities, and acceptance of mycorrhizal inoculants as an alternative to agrochemicals, and
3. Greater emphasis by society toward sustainable agriculture and forestry.

Production of mycorrhizal inoculants is a complex process that requires development of the necessary biotechnological expertise along with related legal, ethical, educational, and commercial requirements.

18.2 Inocula Production of AM Fungi

AM inoculants are marketed today in varied formulations. Some companies market a single strain of mycorrhiza mixed with a carrier. Others sell liquids, powders, and tablets, and most sell cocktails containing a variety of organisms. AM in spore form alone is a poor inoculant; for improved results, AM fungi containing spores, root fragments, and hyphae are superior to those containing only spores. Of greater importance for mycorrhizal inoculants is the degree of infectivity present. Effectivity of an inoculant depends on how rapidly it can colonize the root system. The species used in the inoculant should be effective over a wide range of plant species, pH levels, and soil types. The main obstacle to producing substantial quantities of AM inocula is their obligate nature; this continues to be a major limitation. Two major systems for AM inocula production are (1) soil-based systems and (2) soil-less techniques.

18.2.1 Soil-Based Systems

The inoculum propagation process entails the following stages: (1) isolation of AMF pure culture strain, (2) choice of host plant, and (3) optimization of growing conditions. The soil-based system has been adapted to reproduce different AM strains for increasing propagule numbers in situ (Menge 1984). AM development and its influence on the host are at least partially under genetic control (Gianinazzi-Pearson et al. 1996). Mycorrhizal development is affected by nutrient availability in soil and the inoculum potential of AM fungus. For propagation of AM fungi using the soil-based system, starting fungal inocula usually composed of spores and colonized root segments are incorporated into a growing substrate for plant seedling production (Brundrett et al. 1996). The fungi become established and spread within the substrate and colonize the root seedlings. Both colonized substrates and roots then serve as mycorrhizal inocula. Bagyaraj (1992) found that a mixture of perlite and Soilrite mix (1:1 v/v) was the optimal substrate and *Chloris gayana* (Rhodes grass) the optimal host for mass propagation of mycorrhizal inocula. In addition, pesticides captan and Furadan added to the pot cultures at half the recommended level checked other microbial contaminants with no effect on the mycorrhizal fungi. This technique is very useful for the production of "clean" mycorrhizal inoculum (without other microbial contaminants) with high potentiality in a short span of time.

Douds et al. (2010) have suggested on-farm production of AM fungus to benefit vegetable farmers. perlite-, vermiculite-, and peat-based potting media were tested as diluents of yard clipping compost for media in which the inoculum was produced on *Paspalum notatum* Flugge. All substrates produced satisfactory numbers of AM fungus propagules, though vermiculite proved superior to other potting media (89 vs. 25 propagules cm^{-3}, respectively). Adoption of on-farm production of AM fungal inoculum by growers requires a greater degree of flexibility than that present in the method described earlier (Douds et al. 2006). The original method requires that compost be diluted with vermiculite and that the starter inoculum be in the form of purchased *P. notatum* seedlings colonized by specific isolates of AM fungi. These characteristics are restrictive, particularly the latter. Experiments with perlite-, vermiculite-, and peat-based potting media demonstrated that these restrictions are readily overcome (Douds et al. 2010).

The trap plants commonly used for pot culture of AM fungi are *Sorghum halepense*, *Paspalum notatum*, *Panicum maximum*, *Cenchrus ciliaris*, *Zea mays*, *Trifolium subterraneum*, *Allium cepa*, and *Chloris gayana* (Chellappan et al. 2001; Bagyaraj 1992). The inoculum consists of spores, hyphal segments, and infected root pieces and generally takes 3–4 months to produce on host plants. The practice of pot culture has certain drawbacks that include limited quantities of inocula, bulky nature of inocula, transport problems, risk of contamination, presence of impurities, and lack of genetic stability of inocula (Abdul-Khaliq et al. 2001). Large-scale production of AMF inoculum requires control and optimization of both

host growth and fungal development. The microscopic size of AMF together with complex identification processes contributes to the pitfalls of inoculum propagation.

18.2.2 Soil-Less Techniques

18.2.2.1 Aeroponic Culture

Soil-less culture systems such as aeroponic cultures enable production of spores with limited contamination and facilitate uniform nutrition of colonized plants (Jarstfer and Sylvia 1999). In aeroponic cultures, pure and viable spores of a selected fungus are used to inoculate the cultured plants, which are later transferred into a controlled aeroponic chamber (Singh and Tilak 2001). A fine mist of a well-defined nutrient solution is applied to the roots of the host plant in aeroponic culture. Mycorrhizal cultures have been established successfully using this system (Weathers and Zobel 1992; Mohammad et al. 2000). Three basic methods for producing atomized nutrient solution are as follows:

1. An impeller system making use of an atomizing disk (Zobel et al. 1976),
2. Pressurized spray through nozzles, and
3. Ultrasonically generated fog (Weathers and Zobel 1992).

The fine mist of nutrient solution is required for successful aeroponic culture. Standardization of droplet size is needed so that drops attach to the root system for an adequate time period. Generally, a 45-mm droplet size is optimum; modified Hoagland solution (Epstein 1972) has been used for cultivation of Bahia grass and sweet potato (Wu et al. 1995; Hung and Sylvia 1988). Lack of substrate ensures extensive root growth, colonization and sporulation of the fungus and makes it an ideal system for obtaining sufficient amounts of clean AM fungus propagules (Abdul-Khaliq et al. 2001).

18.2.2.2 Monoxenic Culture

The successful propagation of some AM fungal strains on root organ culture has allowed the cultivation of monoxenic strains that can be used either directly as inoculum or as starting inoculum for large-scale production (Fortin et al. 2002). In vitro bulk production of AMF inoculum is promising, offering clean, viable, contamination-free fungi. The cost of in vitro inoculum may appear prohibitive compared to the cost of greenhouse-propagated inoculum, but its use is a warranty of purity. In vitro production provides research and industry scientists with pure and reliable material for starting inoculum production for both fundamental research and applied technologies (Dalpe 2004). Mass production of AM fungi has

been achieved with several species with increased spore production on monoxenic cultivation. Chabot et al. (1992) produced 25 spores/ml during a 4-month incubation time. St-Arnaud et al. (1996) produced 1,000 spores/ml in 3–4 months. Similarly, Douds (2002) produced 3,250 spores/ml in 7 months, while Adholeya (2003) produced 3,000 spores/ml in 3 months through monoxenic-based inoculum production.

Agrobacterium rhizogenes, a Gram-negative soil bacterium, produces hairy roots and allows roots to grow rapidly on artificial media (Abdul-Khaliq et al. 2001). Once the hairy roots are ready, the collected AM inoculum is surface-sterilized using a suitable surfactant solution. Generally, Tween 20 and a solution choramine T are used for sterilization of AM spores (Fortin et al. 2002). The spores are subsequently rinsed in streptomycin–gentamycin solution (Becard and Piche 1992). The rinsed spores should be stored at 4°C in distilled water or water agar, or on 0.1% $MgSO_4$ $7H_2O$ solidified with gellan gum (Fortin et al. 2002). The nutrient media should be carefully selected to allow growth of the host as well as the fungus. Since roots require rich nutrient medium for growth, AM fungi require a relatively poor nutrient medium (Abdul-Khaliq et al. 2001). Generally, Murashige and Skoog's medium (1962) and White's medium are used for dual culture of host root and AM fungus. Regardless of the high technological investment and high cost, not all AM fungi are successfully culturable in this system. Additionally, the suitability of inoculum produced in vitro, in particular its competitive ability toward other microbes in field soil, has yet to be tested.

18.2.2.3 Nutrient Film Technique

Nutrient film technique (NFT) is a specialized technique developed for commercial production of crops that entails continuous recycling of a large volume of nutrient liquid over a film, which flows over plant roots. The major concern in NFT is the concentration of nutrients. The requirements of nutrient elements vary from one particular mycorrhizal system to another depending upon the size, physiological requirements, and other features of the plants (Sharma et al. 2000). It is necessary to maintain the nutrient solution in the form of a thin film (5 mm to 1 cm). Chemical forms of nutrient elements also affect mycorrhizal infection. Therefore, it is desirable to use a balanced and proper composition.

Low sporulation can be obtained compared to soil-based systems. Problems of contamination by undesirable organisms like rotifers, protozoans, and eelworms are expected because of the common nutrient solution used. The inoculum produced by the NFT method is ideal for the production of easily harvestable solid mats of roots with more concentrated and less bulky forms of inoculum than that produced by plants grown in soil-based or other solid media (Abdul-Khaliq et al. 2001; Chellappan et al. 2001).

18.2.2.4 Polymer-Based Inoculum

It is desirable to apply inoculants to soil with a carrier that can provide physical protection and nutrients for microbial cells (Gentry et al. 2004). For preparation of microbial inoculants, the key issues include microbial selection and characterization, mass production of target microorganisms, selection of carrier material, microbial behavior after formulation, and effectiveness and competitiveness after application (Vassilev et al. 2005).

The simplest method of applying polymer materials is based on the use of hydrogels. Several hydrogels have been used as carriers of AM fungi (Johnson and Hummel 1985; Nemec and Ferguson 1985); however, pH extremes of gel materials have imparted adverse effects on spore germination and root colonization (Vassilev et al. 2005). Entrapment or encapsulation of microbial cells in polymer materials is a highly successful method of immobilization. This method involves entrapment of cells or spores within porous structures, which are formed in situ around the biological material. The carrier should be relatively economical and compatible with the materials that are used for the production of product. The preferred carrier materials include natural polysaccharides and various hydrophilic hydrogels. Various combinations of natural, semisynthetic, and synthetic polymers are available, but the majority incorporates natural polysaccharides including kappa carrageenan, agar, and alginates. Calcium alginates are the most widely used carrier of about 1,350 combinations of carriers in use (Vassilev et al. 2005). The encapsulation of AM fungi produced monoxenically in alginate beads offers the possibility to diversify the inoculation process (Diop 2003). It would be useful to incorporate flavonoids into the capsules (Bécard and Piché 1989; Gianinazzi-Pearson et al. 1989). Some commercially prepared AM inoculants are listed in Table 18.1.

18.2.2.5 Integrated Method

One of the reasons for lower survival and establishment of micropropagated plants during transplantation is the absence of natural associates (Varma and Schuepp 1995). Use of mycorrhizae helper bacteria (MHB) promotes AM symbiosis in various crop plants (Von 1998). The role of MHBs in growth and development of different AM fungi was reported by several workers (Siddiqui and Mahmood 1998; Vosatka et al. 1999). Combined and judicious use of AM fungi and plant-growth-promoting rhizobacteria (PGPR) can provide proper establishment of in vitro propagated plantlets under field conditions. Bhowmik and Singh (2004) reported that PGPR considerably enhanced mycorrhizal colonization and can be used in mass production of AM fungal cultures. da Silva et al. (2007) observed production and infectivity of inoculum of AM fungi multiplied in substrate supplemented with Tris–HCl buffer. Sporulation of AM fungi was also improved in solution with buffer. Large-scale production of inoculum can be obtained by addition of Tris–HCl buffer in nutrient solution and storage at 4°C (da Silva et al. 2007).

Interactions of nitrogen fixers and P-solubilizers with AM fungi have been suggested as one reason for improved growth of many plant species (Turk et al. 2006),

Table 18.1 Commercial AM fungi inoculants produced by different companies

Product	Type of mycorrhiza	Web address for detailed information
AgBio-Endos	Endomycorrhizal inoculant	http://www.agbio-inc.com/agbio-endos.html
Rhizanova™	Endomycorrhizae	http://www.arthurclesen.com/resources/Rhizanova%20Overview%20Sheet.pdf
Bio/Organics	Endomycorrhizal inoculant	http://www.biconet.com/soil/BOmycorrhizae.html
Endorize	Mycorrhizal product	http://www.agron.co.il/en/Endorize.aspx
BuRize	VAM inoculant	http://www.biosci.com/brochure/BRZBro.pdf
Cerakinkong	VA mycorrhizal fungi	http://www.cgc-jp.com/products/microbial/
MYCOgold	AM fungi	http://www.alibaba.com/product/my100200874-100160217-0/Mycogold_Crop_Enhancer_Bio_Fertilizer_.html
BIOGROW Hydo-sol	Endomycorrhizae	http://www.hollandsgiants.com/soil.html
Mycor	Endo/ectomycorrhizae	http://www.planthealthcare.co.uk/pdfs/mycorflyer.pdf
PRO-MIX 'BX'	Endomycorrhizal fungi	http://www.premierhort.com/eProMix/Horticulture/TechnicalData/pdf/TD2-PRO-MIXBX-MYCORISE.pdf
AM 120	Microbial inoculant	http://www.ssseeds.com/other_products.html
BioVAm	Mycorrhizal powder	http://www.harbergraphics.com/Biovam/index.html
Diehard™	Endodrench	http://www.horticulturalalliance.com/DIEHARD_Endo_Drench.asp
MYCOSYM	Mycorrhiza Vitalizer	http://www.mycosym.com/Documents/Flyer%20Olive%20and%20Verticilosis%20WEB.pdf

and these associations are useful in improving survival rates of micropropagated plants (Webster et al. 1995). Microorganisms such as *Frankia*, *Rhizobium,* and *Bradyrhizobium* improve soil-binding capacity, stability, and properties making soil conducive for the establishment of micropropagated plantlets as that of mycorrhiza (Varma and Schuepp 1995).

18.3 Storage of AM Inocula

Propagules of AM fungi must be used immediately once they are extracted or produced. Propagules obtained from soil-less propagation generally have the same requirements for immediate use as those produced in soil-based media. Factors that predispose propagules to higher mortality are harvesting pots, when they are moist, and chopping roots. Chopping roots and mixing contents should be carried out only

just prior to inoculum usage. Conditions for successful long-term storage of AM propagules remain vaguely defined. Spores are generally air-dried and then stored at 4°C. Temperate isolates can be stored at 4–10°C, whereas tropical isolates should be stored at 20–25°C. Feldmann and Idczak (1992) observed that the infectivity of *Glomus etunicatum* stored at 20–23°C and 30–50% relative humidity for 3 years was reduced by only 10–15%.

Fungal viability and mycorrhizal efficiency can be maintained for several months at room temperature (20–25°C), especially when semidry inocula are stored in plastic containers or packaging. Long-term storage (up to 1–2 years) may be conducted at 5°C. More sophisticated and expensive preservation techniques are performed by research institutions. These include the maintenance of inocula on a living plant host grown on sterile growth substrate with regular checks for monospecificity of the cultivated strains, storage in liquid nitrogen (Douds and Schenck 1990), and freeze-drying under vacuum. Kim et al. (2002) reported that cold storage of mixed inoculum enhanced colonization and growth-promoting activity of *G. intraradices* compared to freshly prepared inoculum.

18.4 Inocula Production of Ectomycorrhizal Fungi

The successful application of ECM fungi in plantation forestry depends on the availability of a range of fungi capable of improving the economics of tree production in various environments, and the ability to supply the fungi as inocula (Kuek et al. 1992). Inocula of ECM fungi are usually composed of biomass and carrier material. Many existing or advocated types of inocula only partially satisfy these criteria (Kuek et al. 1992). Three main types of ectomycorrhizal inoculants have been used in nurseries during the last few decades: soil, fungal spores, and vegetative mycelia. Fungal spores obtained from fruiting bodies harvested in natural forests, old nurseries, or established plantations have been used in many parts of the world (Theodorou 1971). They are easy to obtain and apply to plants. Effect of *Scleroderma* on colonization and growth of exotic *Eucalyptus globulus*, *E. urophylla*, *Pinus elliottii*, and *Pinus radiata* was studied (Chen et al. 2006). The results suggest that there is a need to source *Scleroderma* from outside China for inoculating eucalypts in Chinese nurseries, whereas Chinese collections of *Scleroderma* could be used in pine nurseries (Chen et al. 2006).

On the other hand, Lamb and Richards (1974a, b) demonstrated that chlamydospores were less effective than basidiospores as inoculum, and there were significant differences in yield by different fungal species at high inoculum densities and in the presence of added phosphate. Generally, fungal spores are small (ca. 10 μm in length; Clémençon et al. 2004) and are usually produced in large amounts (e.g., 1×10^8 – 1×10^9 spores per sporocarp in *Suillus bovinus*; Dahlberg and Stenlid 1994), enabling long-distance (e.g., intercontinental; Nagarajan and Singh 1990) dispersal by wind or animals (Allen 1991; Ishida et al. 2008). However, basidiospores of most ECM require special environmental conditions for germination,

which are still unknown for many species. Spores of only a few species have been germinated under controlled conditions, a necessary prerequisite to obtain monosporus mycelia to perform mating tests (Martín and Gràcia 2000).

Among ectomycorrhizal basidiomycetes, three main types of germination activators have been reported (Fries 1987): (1) nonectomycorrhizal microorganisms such as colonies of the yeast *Rhodotorula glutinis* (Fries 1976, 1978), the filamentous fungus *Ceratocystis fagacearum* (Oort 1974), and some bacterial isolates obtained from sporophores, mycorrhizae, or soil (Ali and Jackson 1988), (2) a mycelium of the same species as the spores (Fries 1978; Iwase 1992), and (3) roots of higher plants (Melin 1962; Kope and Fortin 1990). Generally, germination activation is caused by some stimulation, such as those from exudates from microorganisms or root exudates. These exudates presumably contain compounds possessing the capacity to trigger spore germination (Kikuchi et al. 2007). Kikuchi et al. (2006) showed that spores of the ectomycorrhizal fungus *Suillus bovinus* germinated through the combination of activated charcoal treatment of media and coculture with seedlings of *Pinus densiflora*. Moreover, they showed that flavonoids play a role as signaling molecules in symbiotic relationships between woody plants and ectomycorrhizal fungi (Kikuchi et al. 2007).

Submerged cultivation of ectomycorrhizal fungi is a convenient technique that has many advantages in relation to solid-state fermentation, viz., a higher viability and biomass productivity, smaller volumes of inoculants, and lower cost compared with other cultivation methods. Inoculant production may be achieved using small bioreactors, and bioreactor cost may be minimized by the adoption of pneumatic reactors such as airlift systems, whose construction and maintenance are less expensive than those of conventional stirred-tank bioreactors. The mycelia produced in submerged culture should be immobilized in alginate gel or other polymeric carriers to maintain viability during storage and after inoculation in the nursery. The application of such alginate-immobilized inoculant is easy and inexpensive.

In order to achieve optimum performance of large-scale bioreactors for inoculant production, it is essential to undertake biochemical and physiological studies of the growth and nutrition of the fungi involved. Only then is it possible to obtain ectomycorrhizal fungal inoculants of high quality at an acceptably low cost and in quantities sufficient to meet the needs of the forest industry (Rossi et al. 2007).

In the production of vegetative ectomycorrhizal inoculants, the selection of mycorrhizal fungi and suitable carrier is important as is the survival and development of inoculant ectomycorrhizal fungi on roots. Techniques for inoculation with pure cultures of selected mycorrhizal fungi have been developed for quasi-operational use by many investigators. Unfortunately, it is the common experience of mycorrhiza researchers worldwide that many mycorrhizal fungi grow poorly or not at all in the pure culture methods attempted thus far. Thus, the practical use of mycelial culture inoculum is limited at present. Fortunately, some of the fungi that grow well in culture have also proven highly beneficial to survival and growth in outplanted stock (Trappe 1977). The ectomycorrhizal fungal genus *Lactarius* has

been intensively marketed in Europe, Asia, and northern Africa, especially the choice edible species *Lactarius deliciosus* and *Lactarius sanguifluus*. *Lactarius* forms ectomycorrhizae with a variety of host plants (Trappe 1962; Hutchison 1999). Some pure culture inoculation studies demonstrate that this species readily colonizes the root system of pines under aseptic conditions. Guerin-Laguette et al. (2000) obtained fruiting body primordia of *L. deliciosus* 1 year after inoculation of *Pinus sylvestris* seedlings in growth pouches and subsequently transferred them to containers. The *L. deliciosus* could be effectively used for controlled mycorrhizal plant production in nurseries as has been successfully done with other ectomycorrhizal fungi. Parladé et al. (2004) described different methods for inoculating seedlings of *Pinus pinaster* and *P. sylvestris* with edible *Lactarius* species under standard greenhouse conditions. All the inoculation methods tested, except the alginate-entrapped mycelium, were appropriate for the production of seedlings colonized with *L. deliciosus*. However, the percentage of colonized plants and the degree of colonization observed were highly variable depending on the inoculation method and the plant-fungal strain combination.

Because of their characteristic odor, flavor, and texture, "matsutake" mushrooms (fruiting bodies or sporocarps) of the ectomycorrhizal fungus *Tricholoma matsutake* are the most sought-after and expensive mushrooms in Japan. Recently, the annual harvest of matsutake mushrooms has declined dramatically as the result of *P. densiflora* trees dying from pine wilt disease caused by the nematode *Bursaphelenchus xylophilus*. Deforestation and modern forestry management practices have also been detrimental to matsutake growth (Wang et al. 1997; Gill et al. 2000). Despite nearly a century of research (Ogawa 1975a, b, 1977), attempts to cultivate matsutake have been unsuccessful. Yamada (1999) reported on the ability of *T. matsutake* isolates to form mycorrhizae using aseptic seedlings of *P. densiflora in vitro*. They germinated pine seeds aseptically on a nutrient agar medium, and pairs of 1-week-old seedlings were transplanted into polymethylpentene bottles containing autoclaved *Sphagnum* moss/vermiculite substrate. The substrate was saturated with nutrient medium containing glucose. At the same time, the bottles were inoculated with a *T. matsutake* isolate. The cultured *T. matsutake* mycelium formed true ectomycorrhizae with *P. densiflora* seedlings in vitro. Moreover, innovative inoculation techniques such as the recent "matsutake sheet" technique (Yoshimura 2004) could be helpful for the inoculation of mature trees in forest ecosystems and could be extended to other late-stage edible mycorrhizal fungi, such as *Boletus edulis*, *Cantharellus cibarius*, and *Amanita caesarea*, which have thus far not been domesticated. Guerin-Laguette et al. (2005) described successful inoculation of mature pine with *T. matsutake* using long root segments (ca. 5–10 mm diameter, 50 cm length) of 50-year-old *Pinus densiflora* trees; the long root segments were excavated, washed, auxin-treated (2–5 mg indole butyric acid, IBA, per root), and incubated in moist *Sphagnum* moss. After 12 months, short roots were regenerated of which approximately 90% were free of mycorrhizae. The mycorrhiza-free short roots were inoculated with mycelial pieces of *T. matsutake* and incubated further in a sterilized substrate. Four-and-a-half months later, roots putatively colonized by matsutake were

sampled near the inoculation points. The authors proposed that the localized inoculation technique was a key step in obtaining early-stage matsutake symbiotic structures *in situ* on a mature tree. Future work should focus on scaling up the inoculation trials *in situ* and on monitoring the persistence of matsutake mycorrhiza (Guerin-Laguette et al. (2005).

18.4.1 Formulation of ECM

In fact, the selection of an appropriate carrier is an important step in the development of a process for inoculant production. The mycelium in the inoculant must remain viable between the time of sowing and the time when receptive roots are formed. The nascent mycelium must resist adverse conditions such as drought, microbial antagonism, or predation by insects and other arthropods (Rossi et al. 2007). In studies to achieve a higher quality of inoculum and an improved production process. Krupa and Piotrowska-Seget (2003) used an alginate-immobilized inoculum of of mycorrhizal fungi to introduce the fubgi to the soil. They reported that the total concentration of cadmium in contaminated soil inoculated with ECM fungi was lower then in non-inoculated soil. As well, Kropáček et al. (1990) reported that they used mycelia of ECM fungi immobilized in alginate gel in a mixture with a silicate carrier-perlite. This inoculum was applied at sowing in forest nurseries to obtian resistant plants for afforestation of areas exposed to man-made stresses. Under both sterile and nonsterile conditions, the growth of seedings and mycorrhiza development were increased by inoculation with a strain Laccaria laccata. These formulation of ECM offers great flexibility as it allows addition of chemical additives to improve gel stability and conserve the inoculant (Mauperin et al. 1987). Inoculant beads can remain viable for several months under refrigeration, although the results vary between fungal species. *Hebeloma westraliense* and *Laccaria laccata* are relatively stable inoculants for more than 5 months; in contrast, the viability of *Elaphomyces* decreased to 40% after 1-month storage (Kuek et al. 1992).

An advantage of alginate gel is the possibility of preparing a multimicrobial inoculant. Douglas fir (*Pseudotsuga menziesii*) seedlings in two bare-root forest nurseries were inoculated with the ectomycorrhizal fungus *L. laccata*, together or not with one of five mycorrhiza helper bacteria isolated from *L. laccata* sporocarps or mycorrhizae and previously selected by in vitro and glasshouse screenings (Duponnois and Garbaye 1991). A dual inoculum composed of calcium alginate beads containing the two microorganisms was a valuable option for increasing the efficiency of ectomycorrhizal inoculation of planting stocks in forest nurseries.

Despite clear evidence from small-scale experiments that ectomycorrhizal fungi improves growth of the host plant, the use of inoculation in plantation forestry is not widespread. In contrast to arbuscular mycorrhizal inoculants, only relatively few ectomycorrhizal fungal inoculants have been commercialized (Table 18.2).

Table 18.2 Commercial ectomycorrhizal fungi inoculants produced through different processes by different companies (Rossi et al. 2007)

Commercial product	Type/process	Company, location
BioGrow Blend®	Spores	Terra Tech, LLC
MycoApply®-Ecto	Spores	Mycorrhizal Applications, Inc.
Mycorise Pro Reclaim®	Propagules ecto+endo	Symbio Technologies, Inc.
Myke® Pro LF3	Propagules	Premier Tech Biotechnologies
Mycor Tree®	Spores	Plant Health Care, Inc.
MycoRhiz®	Mycelium/Solid-state fermentation	Abbott Laboratories
Somycel PV	Mycelium/Solid-state fermentation	INRA-Somycel S.A.
Ectomycorrhiza Spawn	Mycelium/Solid-state fermentation	Sylvan Spawn Laboratory, Inc.
–	Mycelium/Submerged	Rhone Poilenc-INRA
Mycobead®	Mycelium/Submerged	Biosynthetica Pty. Ltd.

18.4.2 Storage of ECM

Ectomycorrhizal fungi are usually maintained by subculturing at approximately 25°C. Ito and Yokoyama (1983) and Jong and Davis (1987) demonstrated that some ectomycorrhizal fungi are preserved by freezing. Corbery and Le Tacon (1997) showed that the survival of ectomycorrhizal fungi after freezing at −196 or −80°C depends on cooling rate and species or strain. The optimum rate of cooling for ECM is −1°C per min. *Thelephora terrestris* and *Paxillus involutus* did not survive any freezing method. The resistance of *Cenococcum geophilum* to freezing may be related to its tolerance to water stress and high salinity. Hung and Molina (1986) reported that, in general, fresh inocula of *Laccaria laccata* and *Hebeloma crustuliniforme* were most effective; their effectiveness remained high for a month of storage and then declined rapidly for a short period, then slowly to the point of no mycorrhiza formation. The effectiveness declined more rapidly with lower inoculation rates. Storage at 2°C prolonged inoculum viability for at least 2 months over that of 21°C storage. Inoculum from different fungal species or isolates within a species responded to storage temperatures differently. *Pisolithus tinctorius* inoculum was the most sensitive: 1-month storage strongly reduced its effectiveness. The difference between 2 and 21°C storage was more obvious in *H. crustuliniforme* than in either isolate of *L. laccata*.

Tibbett et al. (1999) described a method for maintaining viable cultures of ectomycorrhizal *Hebeloma* strains in cold liquid culture medium. Isolates of *Hebeloma* spp., collected over a wide geographic range, were stored at 2°C for 3 years. All cultures survived this storage period and showed a greater time period and success rate than have previously been reported for the long-term storage of ectomycorrhizal basidiomycetes. Rodrigues et al. (1999) studied the viability of fragmented mycelia of *Pisolithus tinctorius* and *Paxillus involutus* entrapped in calcium alginate gel to determine the efficacy of producing ectomycorrhizal fungus inoculum. Fungi were grown in modified Melin-Norkrans (MMN) solution at 28°C before being fragmented in a blender and subsequently entrapped in

calcium alginate. *Paxillus involutus* mycelium was more than 90% viable when entrapped mycelia were 10–50 days old, and *Pisolithus tinctorius* attained its highest viability (55%) for 20- to 40-day-old mycelia. Gel-entrapped *Paxillus involutus* mycelium grew well at all temperatures after 30-day storage, but viability significantly decreased after 60-day storage at 6°C on dry filter paper. For gel-entrapped *Pisolithus tinctorius* mycelia, viability was greatest when stored at 25°C in 0.7 M $CaCl_2$. Entrapment of *Paxillus involutus* fragmented mycelia in calcium alginate beads under the conditions that they propose can be used successfully to produce inoculum. Lehto et al. (2008) grew isolates of *Suillus luteus*, *Suillus variegatus*, *Laccaria laccata*, and *Hebeloma* sp. in liquid culture at room temperature. Subsequently, they exposed samples to a series of temperatures between +5 and −48°C. Relative electrolyte leakage (REL) and regrowth measurements were used to assess damage. The REL test indicated that the lethal temperature for 50% of samples (LT_{50}) was between −8.3 and −13.5°C. However, in the regrowth experiment, all isolates resumed growth after exposure to −8°C and higher temperatures. As high as 64% of *L. laccata* samples, but only 11% in *S. variegatus*, survived at −48°C. There was no growth of *Hebeloma* and *S. luteus* after exposure to −48°C, but part of their samples survived −30°C (Lehto et al. 2008).

Here, we describe inoculant technologies; however, there is currently limited information regarding commercialized products. Therefore, the advent of inoculation technology on a broad scale is necessary, and the overall scientific evidence is important for justifying its use in increasing the economic productivity of forest plantations (Kuek 1994).

18.5 Discussion

Inoculation of plants with mycorrhizal fungi increases the survival and growth rates of seedlings and cuttings in greenhouse and natural conditions. The inoculation also improves the acclimatization of *in vitro* micropropagated plants and promotes earlier flowering and fruiting. These results have arisen because mycorrhizal plants are more efficient in the uptake of specific nutrients and more resistant to diseases caused by soil-borne pathogens. Inoculation of plants with mycorrhizae offers the possibility of reducing fertilizer and pesticide applications. Therefore, mycorrhizal inoculants are gaining popularity as “biofertilizers,” “bioprotectors,” and “biocontrol agents,” and the industry of mycorrhizal inoculum production is expanding worldwide.

To lower the risk of contamination by pathogenic organisms, crops are usually grown in soil-less potting mixes containing different ratios of perlite, vermiculite, peat moss, and composted forest products. Soil-less media also have a lower bulk density and provide better aeration and a higher water-holding capacity than do mineral soils. These artificial rhizosphere conditions may be advantageous to achieve rapid plant growth; however, their effects on mycorrhizal colonization are not well understood. The unpredictability of soil-less media to promote mycorrhizal

colonization can further be confounded by the multiple additives occurring in commercial mycorrhizal inoculants including carriers, fertilizers, humic acid, and soil conditioners. It is necessary to test the infectivity of commonly available commercial mycorrhizal inoculants in standard practices and to analyze plant growth response to inoculation with these products.

Entrepreneurs are currently developing inoculum production systems and marketing mycorrhiza. Still, however, technical difficulties exist for large-scale utilization of mycorrhizal inocula; additionally, numerous legal, ethical, and economical aspects of this technology must be addressed. It is important to fill gaps in fundamental knowledge and to optimize maintenance and application of mycorrhizal fungi in plant production systems. Producers and distributors of inocula should convince users that this technology is economically feasible. More applied studies are needed to aid food and plant production, particularly where sustainable methods of agriculture or horticulture are developing. Moreover, awareness on the part of the public must be encouraged regarding the potential of mycorrhizal technology for sustainable plant production and soil conservation.

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5,

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